Pineal ribbon synapses: regulated by the gland's central innervation

Stefan Reuss

Institute of Microanatomy and Neurobiology, University Medical Center, Johannes Gutenberg-University, Mainz, Germany

Correspondence to: Prof. Dr. Stefan Reuss,
Institute of Microanatomy and Neurobiology,
University Medical Center, Johannes Gutenberg-University,
Langenbeckstr. 1, Bldg. 708, Rm 2.008, 55131 Mainz, Germany.
TEL: +49 6131 178077; FAX: +49 6131 178073; E-MAIL: reuss@uni-mainz.de

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Abstract

OBJECTIVE: The pineal gland is part of the circadian clock system and is under the predominant influence of the endogenous oscillator located in the suprachiasmatic nucleus. A polysynaptic pathway involving hypothalamus, spinal cord and sympathetic system regulates the so far best-studied aspect of its neuroendocrine output, i.e., the synthesis and secretion of melatonin. This parameter increases dramatically at night upon sympathetic activation in rats and many other mammals including man. In addition, parasympathetic, trigeminal, diencephalic and other sites or mechanisms connect the gland, mainly via its stalk, to the nervous system. However, their function for pineal metabolic or morphological features are hardly known. An interesting ultrastructural attribute of the pineal gland are ribbon synapses. These presynaptic structures in pinealocytes are composed of a ribbon and vesicles. They are thought to regulate and facilitate multivesicular release, and display a circadian rhythm with higher levels at night paralleling melatonin synthesis but regulated differently.

METHODS: To gain more insight into the roles of both, the non-sympathetic ("central") innervation and the regulation of pineal ribbon synapses, a surgical transection of the pineal stalk was conducted in rats and the number of synaptic ribbons (SR) were determined by electron microscopy from experimental, sham-operated and control animals.

RESULTS: The transection resulted in normal daytime levels but diminished the nocturnal increase of SR numbers when compared to controls or sham-operated rats.

CONCLUSION: These data provide first evidence that the central innervation of this neuroendocrine organ plays an important role in SR (up)regulation, and that this pathway is antagonistic to the sympathetic innervation.

ORIGINAL ARTICLE

INTRODUCTION

The mammalian pineal gland is part of the circadian clock system and is connected to the endogenous oscillator located in the suprachiasmatic nuclei via multiple polysynaptic pathways. These involve hypothalamic nuclei, spinal cord and sympathetic system, but also parasympathetic, trigeminal and other sites or mechanisms (Klein & Moore 1979, Reuss 2003, Hardeland 2008, Møller & Baeres 2002). These neural inputs are transduced into endocrine outputs (Axelrod 1974), the best-studied aspect of which is the synthesis and secretion of melatonin. In rats, these parameters increase dramatically at night stimulated by noradrenaline released from sympathetic fibers originating in the superior cervical ganglia (SCG; Kappers 1960, Reuss & Moore 1989).

Another pathway thought to influence the gland´s metabolism is the so-called central innervation. Originating in diencephalic (mainly hypothalamic and epithalamic) sites, it reaches the superficial part of the gland via the stria medullaris thalami, the lamina intercalaris (the "deep pineal") and the pineal stalk (Reuss & Møller 1986), bypassing the peripheral sympathetic system. We demonstrated previously that lesioning of this pathway resulted in some alterations of melatonin synthesis (Møller *et al.* 1987, Reuss *et al.* 1987). However, other parameters that may be influenced via this route are unknown.

The present study sought to investigate the possible role of the central pineal innervation on a special morphological feature of the gland. To this end, a surgical transection of the pineal stalk was conducted and the number of synaptic ribbons (SR) was studied. These are presynaptic organelles found in the pineal gland and in sensory cells, and are seen as the key structural specialisation of ribbon synapses (Schmitz 2009). In the pineal, they are located close to the plasma membrane usually apposed to other pinealocytes. Ribbons are shaped as rodlike structures surrounded by clear vesicles and most probably depict high-output synapses releasing more vesicles than conventional synapses. It was hypothesized that retinal SR function as belts that tether vesicles to facilitate coordinated multivesicular release and that they assist high rates of sustained exoxytosis (von Gersdorff 2001, Parsons & Sterling 2003, Sterling & Matthews 2005). Their function in the pineal gland is still obscure. In a recent study (Reuss *et al.* 2010), we found evidence that these structures may also be involved in sensory processes. Synaptic ribbons were identified by their immunoreactivity to the kinesin motor KIF3A, a component of the microtubule motor kinesin II (Muresan *et al.* 1999, tom Dieck *et al.* 2005, Spiwoks-Becker *et al.* 2008). They colocalise the capsaicin receptor TRPV1 that may, for example, be part of mechanisms regulating body temperature via melatonin release from the pineal gland. Indeed, activation of the receptor by capsaicin elicited a clear, dose-dependent augmentation of melatonin secretion (Reuss *et al.* 2010).

A considerable number of studies previously investigated the regulation of pineal SR in response to factors that were thought to influence their numbers in the gland. These are sympathetic and non-sympathetic pathways communicating circadian, seasonal and other influences to the gland. There is evidence that, in particular, non-sympathetic pathways are involved in the regulation of pineal synaptic ribbon expression and function. We therefore sought to investigate the influence of its extra-sympathetic input analyzing SR numbers upon transection of its stalk in rats by transmission electron microscopy.

METHODS

The procedures concerning animals reported in this study complied with German and European laws for the protection of animals and were approved by the county-government office (Bezirksregierung Rheinhessen-Pfalz).

Twenty-six male Sprague-Dawley rats were used for the experiments. They were born and held under a 12:12 h light:dark regimen, at 21±1 °C room temperature with food and water ad libitum. At the age of three months, they were randomly assigned to one of three groups. One group consisted of six rats that were left intact and served as controls. Eight animals belonging to the second group were deeply anesthetized with tribromoethanol (0.3 g/kg b.wt., i.p.) and were subjected to a transection of the pineal stalk (pedunculotomy). We used a modification of a pedunculotomy method described previously (Champney 1989) and the pinealectomy method developed in our laboratory (Pohlmeyer *et al.* 1994). In brief, animals were mounted in a stereotaxic frame (Narishige, Japan). A midline inscission of the scalp was made and a piece of bone removed using a hand-held dental drill. Close to the confluens sinuum, the sagittal sinus was ligated twice and cut between the ligatures. The gland was exposed by lifting the posterior end of the sinus, and gently elevated until the stalk was visible rostral and dorsal to the superior colliculus (see Figure 1A). At a position where it is separated by the suprahabenular recess from the great cerebral vein, the stalk was carefully cut by Yasargil microsurgery scissors. The removed piece of bone was replaced, the skin inscission closed, and the rat was allowed to recover.

The third group was treated just like the pedunculotomy group except the stalk was left intact. After 72 h (daytime) or 60 h (nighttime), the rats were killed by ether overdose. The skull was opened under an operation microscope and the pineals were carefully dissected. A second, unbiased investigator without knowledge of animal treatment and the aim of the study also examined whether or not the stalk was cut through. Only data from animals on which both inves-

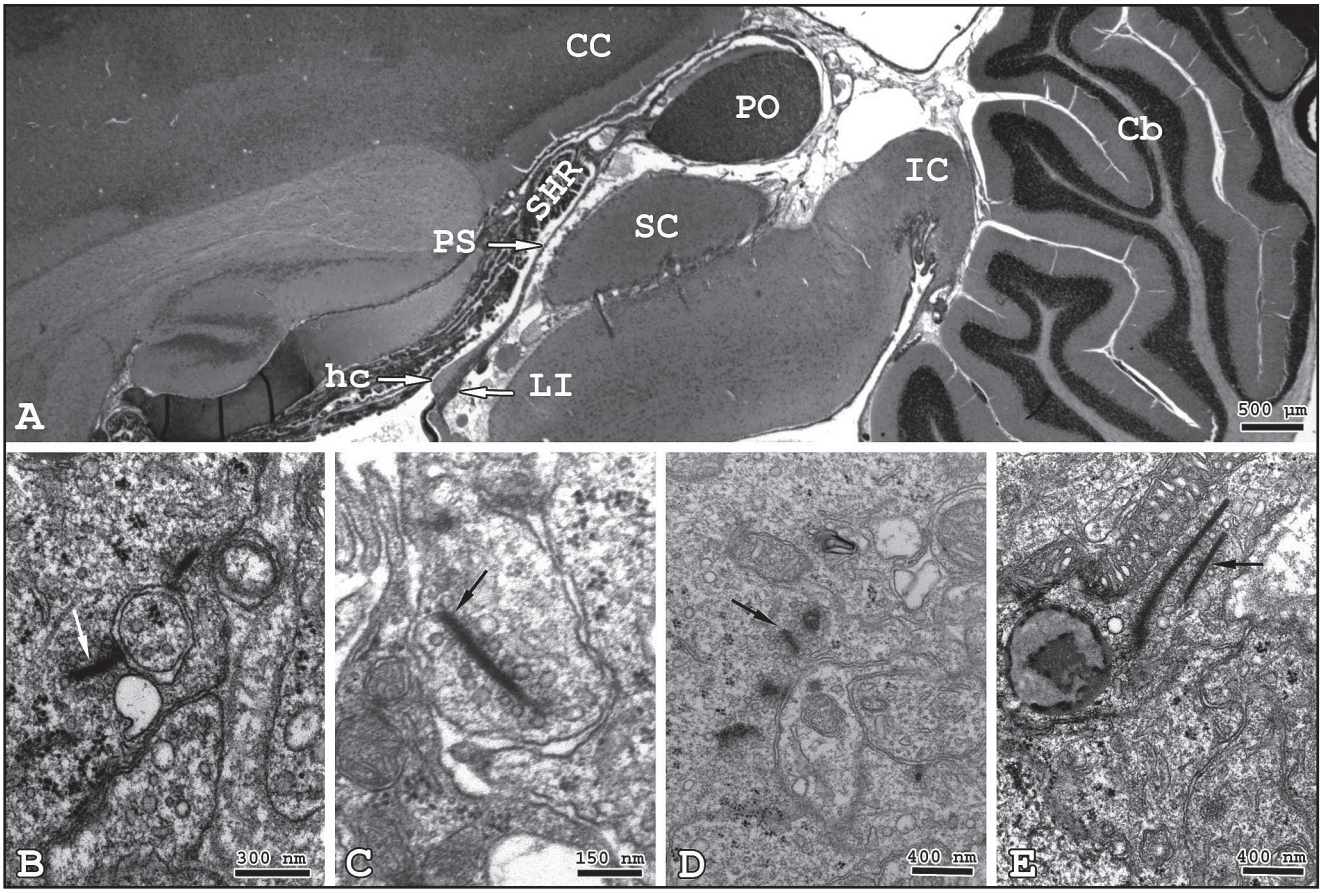

Fig. 1. Structural and ultrastructural aspects of the rat pineal gland. **A**: The midsagittal section shows the superficial part of the pineal gland (PO) as well as its stalk (PS) and deep part (lamina intercalaris, LI). Further abbreviations: Cb cerebellum, CC cerebral cortex, hc habenular commissure, IC inferior colliculus, SC superior colliculus, SHR suprahabenular recess. **B–E**: Synaptic ribbons located near pinealocyte membranes in the superficial pineal gland.

tigators agreed with regard to group affiliation were included. The pineal glands were quickly removed, fixed for 20 h for electron microscopy in a glutaraldehyde-paraformaldehyde solution (Karnovsky 1965), postfixed in 2 % osmiumtetroxide (in 0.2 M cacodylate buffer, pH 7.4) for 1 h, dehydrated in a graded series of acetones, block-stained in 0.8% uranyl acetate and 1% phosphotungstic acid in 70% acetone overnight and embedded in Epon. Ultrathin sections were cut, mounted on uncoated copper grids (300 meshes) and stained with 20 % uranyl acetate in 100% methanol for 1 min, followed by lead citrate for 5 min.

Due to the little thickness of sections (appr. 50 nm) and the size of SR (up to several hundred nm), sectioned profiles of SR were seen in the electron microscope. We will therefore use the term SR profile (SRP). Examination of the sections was performed by an observer unaware of treatment. For the quantitative assessment of SRP, pineal tissue covering six adjacent grid holes was carefully scanned at ×16,000 magnification with a Zeiss EM10 electron microscope. As each grid aperture measured 65 µm by 65 µm, an area of 25,350 µm^2 was examined. To facilitate comparison with other publications, the number of ribbon profiles was converted to 20,000 µm^2. The Wilcoxon-Mann-Whitney U-test and the Kruskal-Wallis H-test were used for statistical evaluation of differences in SRP number between animal groups. The Spearman rank correlation coefficient was computed to test correlation between parameters. A $p<0.05$ is regarded as statistically significant.

RESULTS

As seen in a midsagittal section of the rat brain, the superficial part of the pineal gland is located between cerebral cortex and cerebellum. It is connected to the deep part (lamina intercalaris) by the pineal stalk. Figures 1B–E demonstrate typical synaptic ribbons in pinealocytes from the superficial part of the gland.

Mean daytime numbers of pineal SRP were not affected by treatment, i.e., they were the same in all three groups. Control rats exhibited a clear increase of SRP numbers at night (Figure 2), which was also observed in animals with sham-transection of the stalk ($p<0.01$ in controls, $p<0.05$ in sham-transected rats). In contrast, rats with a transection of the pineal stalk (PS in Figure 1A) showed unaffected daytime levels but their nighttime SRP numbers were clearly reduced as compared to daytime or to controls ($p<0.05$ vs. daytime, $p<0.01$ vs. nighttime controls/sham transected rats).

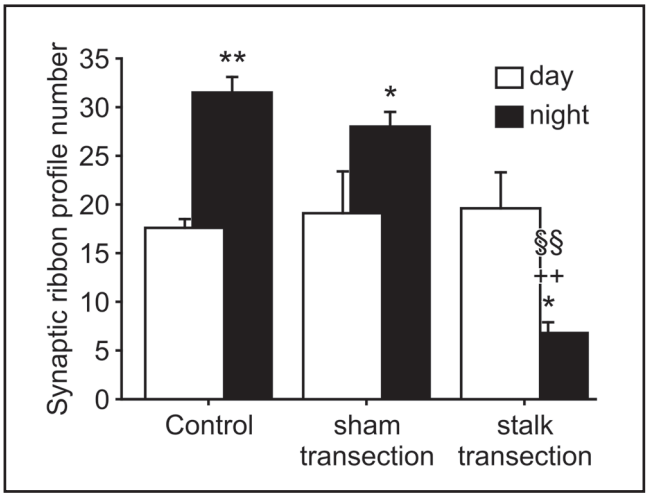

Fig. 2. Central regulation of synaptic ribbon expression. Number of synaptic ribbons profiles in the rat pineal gland at day and night in untreated control rats, sham-operated animals and in stalk-transected rats (mean ± SEM/20,000 μm^2, n=7). Statistically significant differences are indicated (* $p<0.05$, ** $p<0.01$ nighttime vs. daytime of the same group, $^{++}$ $p<0.01$ stalk transection vs. controls; §§ $p<0.01$ stalk transection vs. sham transection, nighttime.

DISCUSSION

Our present rat data demonstrate that pineal ribbon synapses are dynamic structures that respond to experimental manipulations of the gland´s nervous input. This fits well with physiological changes in SR numbers and with the view that the its protein composition may change during the 24 h-cycle in rat pineals (Spiwoks-Becker *et al.* 2008). It is thought that they provide a steady supply of synaptic vesicles for the continuous release of transmitters, but are also important for the transient, synchronous release (LoGiudice & Matthews 2009; Schmitz 2009).

The nocturnal increase of SRP numbers observed in our control groups is in accord with the literature and had been previously found in several mammalian species including the rat (Vollrath 1973, Matsushima *et al.* 1983). This increase was primarily thought to be due to adrenergic mechanisms, as it parallels the sympathetically-driven nocturnal elevations of pineal electrical and metabolic activities. However, sympathetic denervation by surgical or chemical ganglionectomy diminished electrical and abolished metabolic pineal rhythms while it augmented SR numbers (Reiter *et al.* 1979, Reuss 1986, King & Dougherty 1982, Reuss 1989, Karasek *et al.* 1982), leaving the impression that the sympathetic impact dampens SR proliferation.

This view is supported by the previous finding that SRP numbers are inversely proportional to the density of adrenergic nerve endings and to norepinephrine concentration in the pineal gland (Karasek *et al.* 1983) and by our recent immunohistochemical data which show that rich tyrosine hydroxylase-staining is associated with low SRP numbers and vice versa (Reuss *et al.* 2010). The SR may function in communication processes between adjacent pinealocytes. It seems that their number is high in regions with (or situations of) less sympathetic influence and lower where (or when) sufficient noradrenergic impact is present. This would, however, lead to lower nighttime numbers of SRP, which is definitely not the case in mammals studied.

Now the question arises as to which structure – if not the sympathetic system – upregulates SRP numbers at night? Major candidates are inputs to the gland that originate in parasympathetic, trigeminal or diencephalic sites such as habenular and hypothalamic paraventricular or mamillary regions (Reuss 1999, Møller & Baeres 2002). Tracing experiments and immunocytochemical studies have shown that nervous fibers enter the deep pineal via the stria medullaris thalami and may reach the superficial part of the gland through its stalk (Matsushima *et al.* 1999, Sakai *et al.* 2001, Møller & Baeres 2002, Reuss & Møller 1986). Lesioning of this pathway led to alterations of melatonin synthesis including augmented early morning levels (Møller *et al.* 1987, Reuss *et al.* 1987). These and other data support the hypothesis of a complex interaction of peripheral sympathetic as well as central noradrenergic and non-noradrenergic (e.g., cholinergic) mechanisms in regulating pineal melatonin synthesis (Reuss *et al.* 1992, Hardeland 2008). The widely open roles of both the central innervation and the regulation of pineal SR warranted the present study, i.e., to disconnect this pathway and to study possible effects on pineal SRP numbers.

The data now show that in rats subjected to transection of the pineal stalk, daytime SPR numbers were not affected but nighttime levels were diminished well below daytime numbers when compared to control and sham-operated animals. This first demonstration of experimentally lowered nocturnal SRP numbers points to an extrasympathetic influence on the expression of ribbon synapses.

Considering the present and previous sympathectomy data, the following mechanism may be regarded as a working hypothesis. Nocturnal SRP numbers are regulated to a distinct level by a central influence that forces up SRP numbers, and a sympathetic influence that sets upper limits. Abolishing the central influence by pedunculotomy neutralises the nocturnal increase (this study), and abolishing the sympathetic influence compensates for the limitation of SRP numbers´ nocturnal increase. It may thus be speculated that pineal intercellular communication is enhanced at night, and when or where sympathetic influence is attenuated, and that numerically increased ribbon synapses are the ultrastructural correlate for this in the neuroendocrine organ.

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