J. Pineal Res. 2011; 51:17–43
Doi:10.1111/j.1600-079X.2011.00856.x

Journal of Pineal Research

Journal of Pineal Research
Molecular, Biological, Physiological and Clinical Aspects of Melatonin

REVIEW ARTICLE

A survey of molecular details in the human pineal gland in the light of phylogeny, structure, function and chronobiological diseases

Abstract: The human pineal gland is a neuroendocrine transducer that forms an integral part of the brain. Through the nocturnally elevated synthesis and release of the neurohormone melatonin, the pineal gland encodes and disseminates information on circadian time, thus coupling the outside world to the biochemical and physiological internal demands of the body. Approaches to better understand molecular details behind the rhythmic signalling in the human pineal gland are limited but implicitly warranted, as human chronobiological dysfunctions are often associated with alterations in melatonin synthesis. Current knowledge on melatonin synthesis in the human pineal gland is based on minimally invasive analyses, and by the comparison of signalling events between different vertebrate species, with emphasis put on data acquired in sheep and other primates. Together with investigations using autoptic pineal tissue, a remnant silhouette of premortem dynamics within the hormone's biosynthesis pathway can be constructed. The detected biochemical scenario behind the generation of dynamics in melatonin synthesis positions the human pineal gland surprisingly isolated. In this neuroendocrine brain structure, protein-protein interactions and nucleo-cytoplasmic protein shuttling indicate furthermore a novel twist in the molecular dynamics in the cells of this neuroendocrine brain structure. These findings have to be seen in the light that an impaired melatonin synthesis is observed in elderly and/or demented patients, in individuals affected by Alzheimer's disease, Smith–Magenis syndrome, autism spectrum disorder and sleep phase disorders. Already, recent advances in understanding signalling dynamics in the human pineal gland have significantly helped to counteract chronobiological dysfunctions through a proper restoration of the nocturnal melatonin surge.

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Key words: acetylserotonin *O*-methyltransferase, arylalkylamine *N*-acetyltransferase, autoptic brain tissue, chronobiology, clock genes, melatonin, neuroendocrine transducer

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Received October 20, 2010;
Accepted December 16, 2010.

Introduction and historical perspectives

One of the very early fathers of human brain anatomy, the Greek physician Herophilos (335-280 B.C.), noticed the close topographical location of the human pineal gland (*glandula pinealis; corpus pineale; epiphysis cerebri*) to the bottlenecks of the inner liquor system, namely the third ventricle, the *aquaeductus mesencephali Sylvii* and the *aquaeductus laterales Monroi*. He therefore considered this enigmatic brain structure to function as a sluice to regulate the flow of both liquor and mind flux. Detailed anatomical investigations into the topography of the human pineal gland were made by Andreas Vesalius Bruxellensis (1514–1564) during the Renaissance period. These early anatomical descriptions led René Descartes (1596–1650 A.C.) to postulate that the pineal gland is a reservoir for the spirits of life (*spiritus animalis*), but he also considered this brain structure as a transducer of visual inputs into neuronal signals. Although the latter interpretation emerged some 400 yr ago and was based on visual inspection of the human brain, it surprisingly converges with our current knowledge on multisynaptic connections from the eyes to the pineal gland.

Today, we have learned from fundamental experiments conducted during the first half of the last century [1–4] that the *glandula pinealis* is more but a phylogenetic rudiment of the so-called 'third eye', or the parietal eye of lower vertebrates, but that it functions in general in all vertebrates as a neuroendocrine transducer. We currently know that depending on the species the pineal gland can either directly or indirectly convert environmental lighting conditions into a neurohormonal message, the nocturnally elevated synthesis of melatonin [5–11].

In lower vertebrates, the timed gating of melatonin production is linked to the direct photosensitivity of pinealocytes resulting in light-mediated inhibition of

melatonin synthesis [12]. The mammalian pineal gland, on the other hand, lost through the course of evolution this direct photosensitivity, and consequently, rhythmic melatonin synthesis relies on rhythmic neuronal inputs from a circadian (*circa*: about; *dies*: day) master clock that resides in the hypothalamic suprachiasmatic nucleus (SCN) [13–16]. In mammals, the brain structures responsible for sensing environmental light directly and indirectly, namely the retina, the SCN and the pineal gland, respectively, are united in the so-called photoneuroendocrine system [7] (Fig. 1).

Major advances to unravel the function of the human pineal gland were achieved during the second half of the 20th century, when melatonin was extracted from bovine pineals, and showed an antagonizing effect of the melanocyte-stimulating hormone (MSH) in *Xenopus* dermal melanophores [17]. This functional role of melatonin is lost during the course of evolution in mammals.

In contrast to hormone detention in the vertebrate pituitary, current knowledge does not favour the storage of newly synthesized melatonin in pinealocytes. Rather, upon nighttime-restricted synthesis, the highly lipophilic melatonin is rapidly translocated across biological membranes and released into the circulation [7, 10] to reach its remote targets.

A second breakthrough in understanding the physiological importance of the pineal hormone in mammals was the

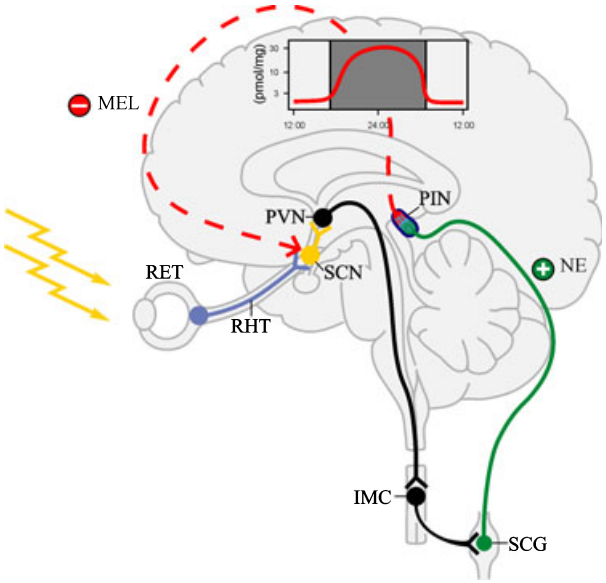

Fig. 1. The photoneuroendocrine system in the human. The photoneuroendocrine system consists of the retina (RET) that perceives environmental light information, the retinohypothalamic tract (RHT) that transmits light signals to the suprachiasmatic nuclei (SCN), which constitutes the site of the endogenous circadian oscillator. SCN-efferent circadian cues are transmitted via the paraventricular nuclei (PVN), the intermediolateral column of the spinal cord (IMC) to the superior cervical ganglia (SCG). Postganglionic sympathetic fibres stimulate through norepinephrine the nocturnal increase in melatonin synthesis (MEL). MEL can then feed back on the endogenous clock. The inset shows schematically dynamics of melatonin synthesis in the human pineal gland, with the shaded area representing the night (modified with permission from [8]).

cloning, and subsequent functional characterization, of mammalian G-protein-coupled melatonin receptors (MT_1, MT_2), expressed in the brain and in the pituitary [18–22]. In situ hybridization studies showed a low but widespread distribution of MT_1 mRNA in the human brain [23–25], but no specific signals were detected so far using an MT_2 cDNA probe [18]. Consequently, molecular interactions between the pineal hormone and intracellular signalling molecules were investigated. Melatonin receptor–mediated transduction of the melatonin signal was shown to occur via parallel pathways, involving inhibition of adenylyl cyclase and stimulation of phospholipase C [26–28].

In addition, a third putative melatonin-binding site was identified, the enzyme quinone reductase 2 [29, 30], with melatonin possibly serving as a cosubstrate. The subsequently characterized binding affinity of the QR2 enzyme for melatonin and for specific ligands opened the way to potentially better understand the critically debated antioxidative and neuroprotective potency of the pineal hormone [31]. Little is known, however, about the distribution of QR2 in the primate brain, its possible function(s) and signalling events involved in melatonin's action as a QR2 inhibitor [32]. GPR50, an orphan melatonin-related G-protein-coupled receptor, was stipulated to be the mammalian orthologue of the nonmammalian melatonin receptor, Mel 1c [33], with genetic evidence emerging that it may play a role in psychiatric disorders, like mental illnesses, but also in metabolic sensing [34–36]. GPR50 shares 45% homology with MT_1 and MT_2 transmembrane domains but does not bind melatonin [19]. In vitro experiments suggested a ligand-independent modulation of melatonin pharmacological effects through dimerization of GPR50 with MT_1 and/or MT_2 [31].

Delineating the anatomical location of melatonin-binding sites in the vertebrate brain demonstrated a large variation between species, but an almost consistent presence in the SCN [16, 37, 38]. While melatonin modulates various physiological parameters such as seasonal reproduction, vascular tone, immune function and possibly also tumour cell growth [39, 40], its ability to potentially phase shift the SCN clockwork is its central and the best characterized chronobiological effect in human physiology [39, 41–43].

This review focuses on integrating the known structure–function relationship of the human pineal gland with the recently acquired knowledge on the molecular level of this organ. Morphology, anatomy and vasculature of the human pineal gland are only briefly reviewed (see section: Anatomy and topography of the human pineal gland), as there exists extensive and excellent literature on these topics [7, 10, 44–46].

Dynamics and physiological importance of melatonin synthesis in lower vertebrates and nonhuman mammals are well investigated (see section: Melatonin synthesis in the vertebrate pineal gland: The nonmammalian pineal gland) and will be touched briefly only to next point out the link between melatonin signalling dynamics in pinealocytes and its systemic role in human circadian physiology (see section: Human).

The fact that in humans melatonin can only be measured through noninvasive or minimal invasive techniques in

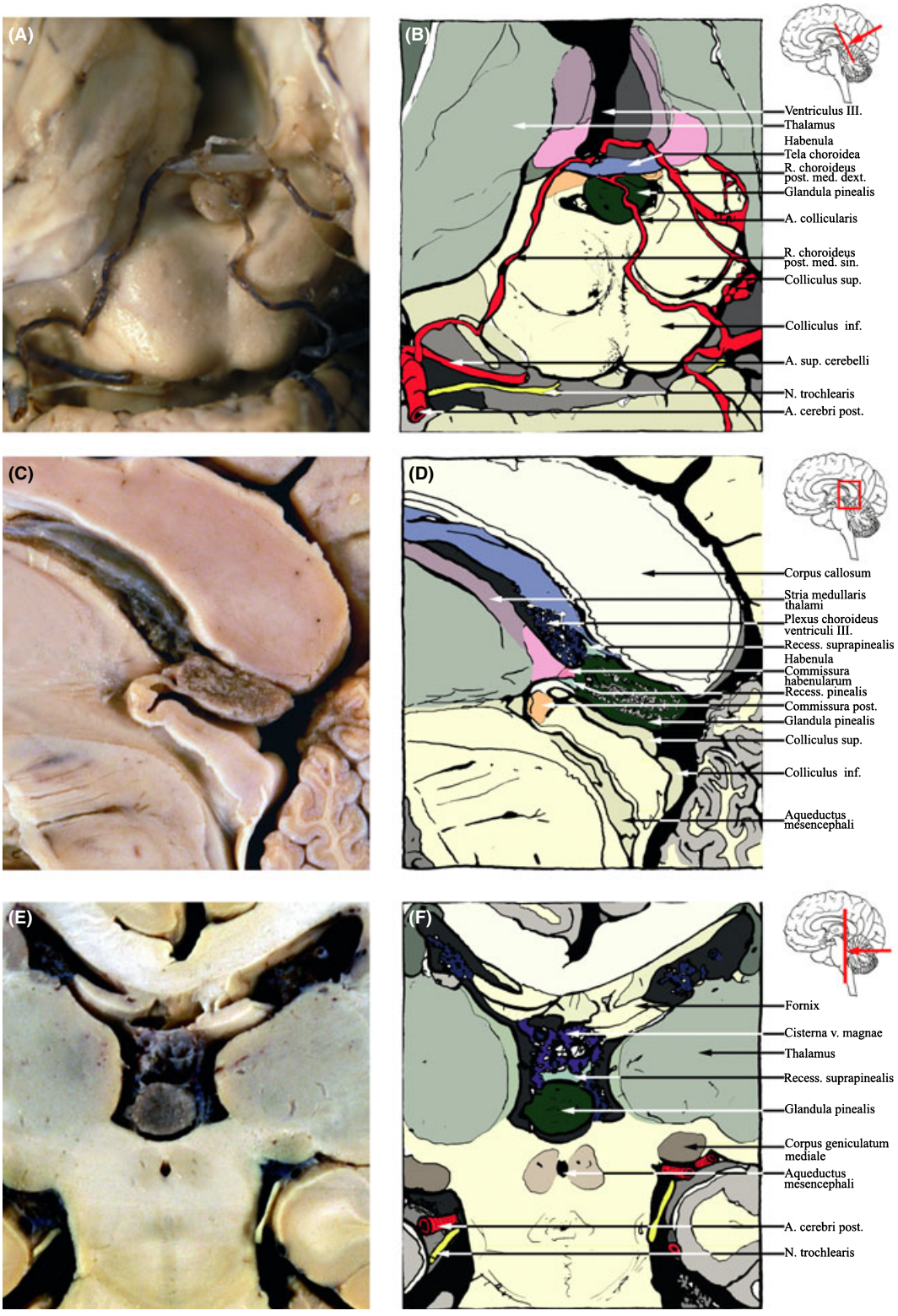

Fig. 2. Topographical anatomy of the human pineal gland (*glandula pinealis*). Photographic pictures of unfixed and unstained human brains, demonstrating in situ the topografic localization of the pineal gland and neighbouring vascularization. Original pictures are shown in left columns of images (A, C, E, G, I, L), and corresponding drawings of images with visible structures indicated and named are shown in right columns (B, D, F, H, K, M). The red arrow in schematically drawn whole brain image shown in the upper right corners of each pair of pictures indicates direction of inspection. For better visualization of the human pineal gland, parts of the brain have been removed. Anatomical denominations were carried out according to the *Nomina anatomica*.

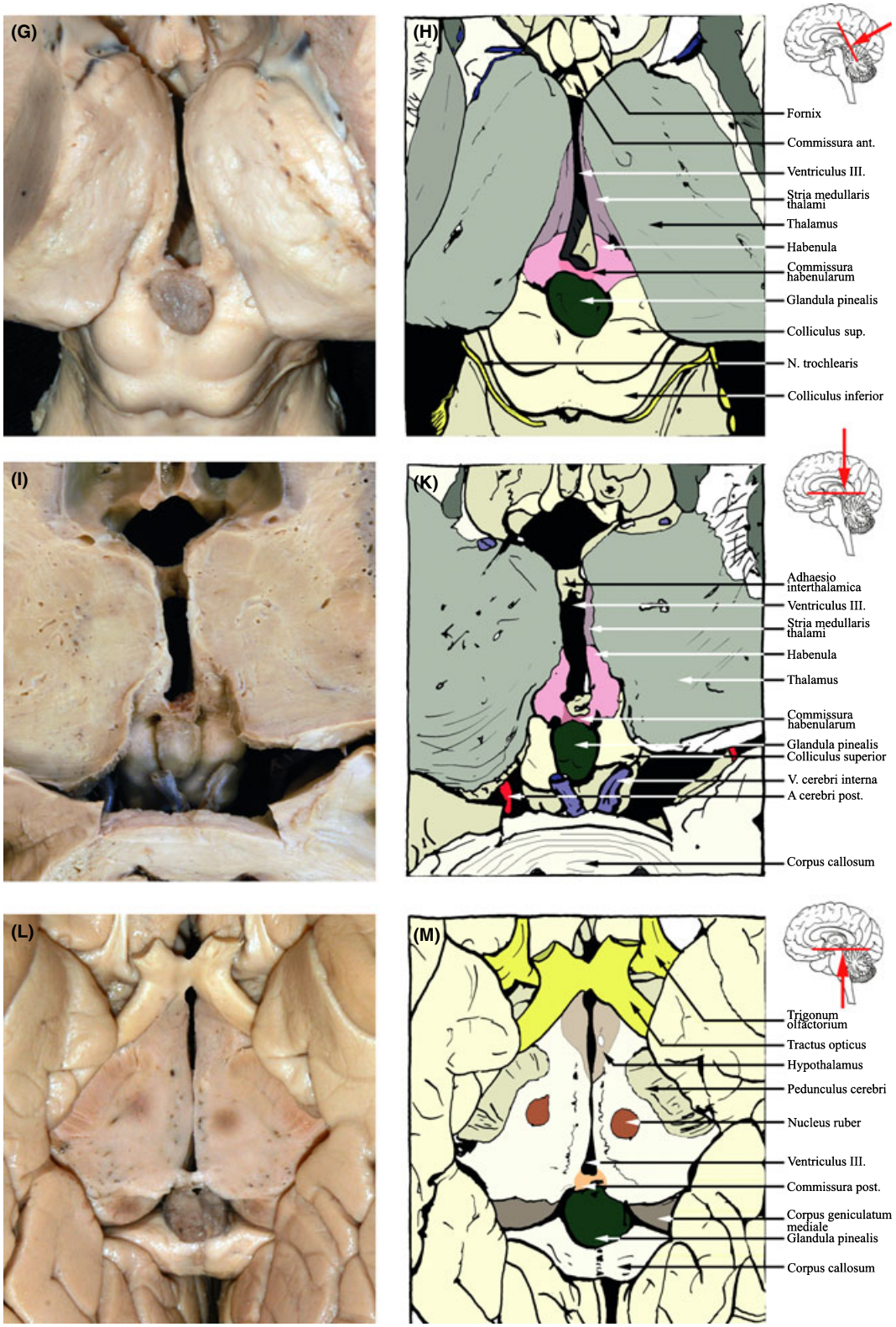

Fig. 2. (Continued)

body fluids only [39, 47] leaves a gap in the knowledge about the biochemical mechanisms behind the generation of the rhythmic hormonal signal. The review will therefore integrate recently achieved data on signalling events, gained by using autoptic brain material (see section: Molecular details of human melatonin synthesis).

To cladistically position this neuroendocrine structure, pineal signalling mechanisms are compared between the human and other well-investigated mammalian species (see section: Melatonin synthesis in the vertebrate pineal gland and Clock gene expression in the vertebrate pineal gland).

While the consequences of a disturbed melatonin synthesis by environmental factors ('social jet lag', shift work, blindness, SAD, jet lag) are only briefly mentioned (for extensive reviews see [39, 42, 43, 48]), some pathophysiological melatonin rhythms in the human that are causatively related to an aberrant pineal signalling will be presented.

Known molecular details behind these chronobiological dysfunctions will be addressed in this review, as they are associated partly with dramatic effects on human behaviour (section Disturbed melatonin synthesis, causes and curative countermeasures).

References to nonhuman literature and details on experimentally gained insights into signalling in the pineal gland of rodents and ungulates are given, when considered appropriate.

An additional emphasis is put on data reported in sheep, as this species shows a high degree of similarity in pineal anatomy and biochemistry, when compared with human.

The rare results obtained experimentally in primates are included in this review to finally discuss the novel finding of clock gene expression in mammalian pinealocytes and particularly in human, with respect to their possible role within the generation and maintenance of rhythmic processes (section Clock gene expression in the vertebrate pineal gland).

Anatomy and topography of the human pineal gland

In all vertebrates, including human, the pineal gland is a derivative of the diencephalic roof plate. The human *glandula pinealis* occupies the impression between the superior colliculi of the mesencephalon and is burrowed between the two hemispheres (Fig. 2). The third ventricle penetrates as an evagination into the proximal part of the pineal gland (the so-called pineal recess) and divides the pineal stalk into an inferior lamina, comprising the posterior commissure, and the superior lamina, housing the habenular commissure (Fig. 2C,D,G–K). The piriform-shaped organ can often be easily distinguished from the surrounding brain matter based on visual distinctions in colour (Fig. 2).

The general anatomical and histological details of the human *glandula pinealis* have been described earlier [1, 10]. Briefly, the human pineal gland belongs to the proximal or type-A pineal glands (classification of Vollrath [10]), as both an intermediate part and a superficial part of the organ are missing (Fig. 2C,D). The bulk of the pineal tissue lies in close proximity to the dorsal aspects of the human diencephalon and borders the third ventricle (Fig. 2; anatomical preparations of unfixed and unstained human autoptic brains were conducted in accordance with the Declaration of Helsinki and approved by the local Ethics Commission of the University Clinics, Frankfurt/Main, Germany).

As the demonstration that silver nitrate in the drinking water led to the deposit of silver in the close vicinity of pineal blood vessels in rats, it has been commonly believed that the human pineal gland also lacks a blood–brain barrier like other circumventricular organs [10, 49]. The organ receives a very rich arterial blood supply, with details elaborated and shown here (Fig. 2)[44]. The medial and the lateral branches of the posterior choroidal arteries (*Aa. choroideae posteriores)*, originating from the two P2-segments, postcommunicating part (*pars postcommunicalis*) of the posterior cerebral arteries (*Aa. cerebri posteriores),* supply the organ with blood (Fig. 2A,E,I). Rarely, branches of the collicular artery (*A. collicularis)* are also involved (Fig. 2A). Before entering the pineal parenchyma, the small intrapineal arterial branches run inside the *pia mater*. The topographical vicinity of the human pineal gland to the great cerebral vein (*V. cerebri magna*, also known as Galens vein) and the internal cerebral veins (*Vv. cerebri internae)* is suggestive of their involvement in the drainage of pineal secretory products, via the internal jugular veins (*V. jugulares interna*) (Fig. 2I,K). Indeed, it was shown in sheep that the amount of melatonin in the jugular veins mirrors the nocturnally elevated concentration of the pineal hormone in the cerebrospinal fluid (CSF; *liquor cerebrospinalis*) and thereby accurately reflects the duration of the night [50]. On the basis of these data and because of the comparable topographical location of the pineal gland in humans, it has to be assumed that melatonin is released into two compartments, namely into the CSF to affect brain structures and into the blood stream to affect peripheral organs.

Next to the intimate contact of the human pineal gland with the brain's venous drainage system, the proximal part of the human pineal gland is separated from the pineal and suprapineal recesses (*recessus pinealis et suprapinealis)* of the ventricular system (Fig. 2C,E) by only an ependymal lining, covering the pineal parenchyma. Moreover, the outer close aspects of the human pineal gland are 'bathed' in the CSF (Fig. 2).

On the basis of the here described anatomical location, it was also suggested that melatonin is released not only into the circulation but also into the CSF [10, 50]. This suggestion was initially confirmed when melatonin was detected in human CSF samples taken during postmortem autopsies [51]. Subsequently, it was observed that the daytime-dependent differences in melatonin content in human autoptic pineal tissue paralleled the alterations in melatonin levels in both serum and CSF (Fig. 3), with higher concentrations in the CSF [51–54]. The use of the in vivo endoscopic microsampling technique demonstrated decreased melatonin concentrations with increased distance of the ventricular sampling place to the pineal gland in the human [55]. Additional experimental support for a melatonin release into the CSF was obtained in sheep. An elegant in vivo study, using intraventricular cannula implantations, reported that within the third ventricle,

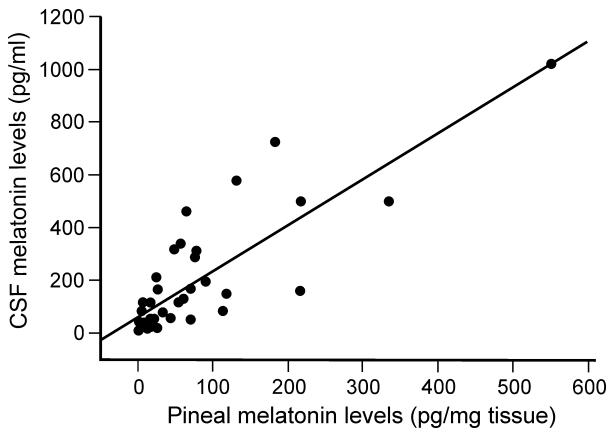

Fig. 3. Melatonin levels in the pineal gland and cerebrospinal fluid (CSF) of human individuals. Existence of a significant positive correlation (regression slope: 0.83, $P < 0.0001$) between melatonin levels, as assessed postmortem in the CSF and in the pineal gland derived from 41 subjects, indicating that CSF alterations in melatonin levels reflect changes in pineal melatonin content (modified with permission from [52]).

nighttime melatonin concentrations are more than 100-fold higher close to the pineal recess, when compared with the ventral part of the ventricular system [56]. These observations suggest a remarkable hormone release into the CSF in sheep, which likely resembles the situation in human.

From these results, it may be suggested that melatonin serves as an endocrine signalling molecule, functioning in intracerebral information flow between different brain areas in mammals, including the human, although it also may be possible that melatonin, present in the CSF, represents only a spillover. However, the physiologically important function of melatonin, carried along in the CSF, is underlined in mammals by the presence of high-affinity melatonin receptors in close vicinity of the third ventricle, which is particularly true for neurons in the human SCN [20, 37, 38, 57–59]. In fact, the highly lipophilic characteristic of melatonin allows targeting of remote brain cells and neurons that do not necessarily have contact with the liquor but are reached by the hormone through diffusion.

Melatonin synthesis in the vertebrate pineal gland

Signalling pathways involved in melatonin synthesis are well investigated in many vertebrate species [6, 7]. Common to all vertebrate phyla, melatonin synthesis is initiated with the active uptake of the amino acid tryptophan into pinealocytes, where it is converted by the tryptophan hydroxylase into 5-hydroxytryptophan and thereafter decarboxylated by the aromatic amino acid decarboxylase to become 5-hydroxytryptamine (5-HT/serotonin). The subsequent *N*-acetylation of the molecule by the arylalkylamine *N*-acetyltransferase (AANAT) is considered to be the rate-limiting step in melatonin synthesis, driven by the nocturnally elevated release of the neurotransmitter norepinephrine (NE) from the sympathetic innervation to the pineal gland. Eventually, *N*-acetylserotonin is converted by the acetylserotonin *O*-methyltransferase (ASMT; also termed hydroxyindole *O*-methyltransferase [HIOMT]) into melatonin [6, 7]. It should be noted that ASMT, the final enzyme in melatonin synthesis, may play a more important role than assumed so far, as modulation of AANAT activity over a wide dynamic range is not followed by parallel changes in melatonin synthesis [60, 61]. In particular, it has been shown in the Syrian hamster pineal gland that ASMT may be important to control photoperiodic variations in the amplitude of the nocturnal melatonin peak [60].

Despite this common and highly preserved biochemical pathway for melatonin synthesis in all vertebrate species investigated so far, there exist remarkable differences in the regulation of melatonin synthesis and the involved signalling pathways. Understanding these dynamics in animal models may allow prediction of the biochemical dynamics in the human pineal gland.

The nonmammalian pineal gland

In nonmammalian vertebrates, pinealocytes are still directly photosensitive [7]. In anamniotes, like fish and amphibians, pinealocytes with retinal cone photoreceptor-like properties predominate, while in sauropsids, like reptiles and birds, photoreceptor properties are reduced [62]. In both anamniotes and sauropsides, the direct photic input to the pineal gland adjusts ('phase-shifts') and gates melatonin synthesis. Moreover, pinealocytes of lower vertebrates anticipate the daily cycling in lighting conditions through the inheritance of endogenous circadian clockwork [63, 64]. Thus, nonmammalian pinealocytes house the complete machinery of an autonomous circadian clock such that the 'message of darkness' can be re-created in an in vitro dish [63].

The mammalian pineal gland

In the mammalian brain, a remarkable separation of tasks for visual inputs evolved (Fig. 1). Owing to a developmental re-programming [8, 62], lateral eyes function as inputs for the discrimination of shape and colour of objects. Melanopsin-positive retinal ganglion cells filter the circadian information from the environmental light and entrain the SCN via the retino-hypothalamic tract to external lighting conditions [65, 66]. The anatomical characterization of the retinal-entraining pathways has been shown in various animal models, including the Bonnet macaques as a primate [65]. Consistent results, obtained from retinal tract-tracing studies in various mammalian species, indicate the existence of distinct retinal projections to the ventral and ventrolateral subdivision of the SCN also in humans [65, 67].

In photoperiodic species, like hamster and sheep, the duration of the nocturnal melatonin signal is determined by seasonal variations in nighttime durations and consequently functions as a major regulator of seasonal reproduction, with pinealectomy abolishing this physiological response [68–70]. The involvement of melatonin in seasonal reproduction has also been clearly shown in nonhuman primates (*Macaca mulatta*) [71, 72]. Even in humans, a careful analysis of annual conception reveals a masked seasonality, when taking the shielding of body function from seasonal cues in industrialized countries into account, [73–78].

Disrupting SCN signalling in the mammalian pineal gland via ablation of the central clock, pharmacological blocking of the stimulatory NE input, dissection of the sympathetic innervations or even culturing the pineal gland in vitro leads to a rapid disappearance of parameters that display a strong circadian rhythm in vivo, i.e. melatonin synthesis, serotonin release or transcription of cAMP-inducible genes like *Aanat*, *Asmt*, *Period1* or *Icer* [8, 79].

Despite the superior importance of the NE input, various neuroanatomical and immunohistochemical investigations revealed a rich diversity in additional neuronal, endocrine and paracrine inputs to the mammalian pineal gland. In the rodent pineal gland, a multitude of neurotransmitters, such as peptides, monoamines, aminoacids, steroids and even gaseous transmitters, have been shown to modulate melatonin synthesis (for comprehensive reviews, see: [70, 80–82]).

In the monkey pineal gland, an immunoreaction for neuropeptide Y [83], substance P and Lutheinizing hormone releasing hormon [84] was demonstrated, which may account for a possible modulatory function on the NE-induced melatonin synthesis. While it may be assumed that a similar diversity in signalling mechanisms may be present in the human pineal gland, only an immunoreaction for enkephalin [85] and somatostatin [86] has been described so far.

Comparative physiological investigations into the complex input pathways in pineal signalling demonstrated remarkable species-specific differences in the regulation of hormone synthesis [87–89]. Hormone production in rodents only starts several hours after darkness and shows an anticipatory offset at the end of the night [90], while in sheep, primates and humans, a rapid onset of melatonin production is observed at dusk (dim light melatonin onset [DLMO]) [91, 92], which is terminated at dawn with the appearance of light [93, 94]. The DLMO is defined as the interpolated daytime, after which melatonin levels exceed the average of its lowest value by 25%, which can be predicted, provided individuals are exposed to not more than ten lux of light intensity during consecutive nighttime sample collections [92].

The above-mentioned observations indicate that similar or even identical biochemical steps are used in rodents and primates to generate the melatonin rhythm pattern. However, with results obtained in nonhuman mammalian species significantly helped to predict the signalling routes in the human pineal gland. The analysis of regulatory routes using autoptic human tissue has described remarkable differences in mechanisms to generate the nocturnal melatonin surge (see section Human).

Rodents

In rodents, the experimentally deciphered molecular details behind the dynamics in rhythmic melatonin synthesis showed that it emerges from a core cAMP-dependent transcriptional control of the penultimate enzyme, the AANAT [6, 15, 88, 89, 95]. The activation of the cAMP-signalling pathway by the elevated release of NE from sympathetic nerve fibres leads to a 100-fold increase in the amount of *Aanat* mRNA and to a manifold increase in AANAT protein and activity [96, 97] (Fig. 4, Table 1). This

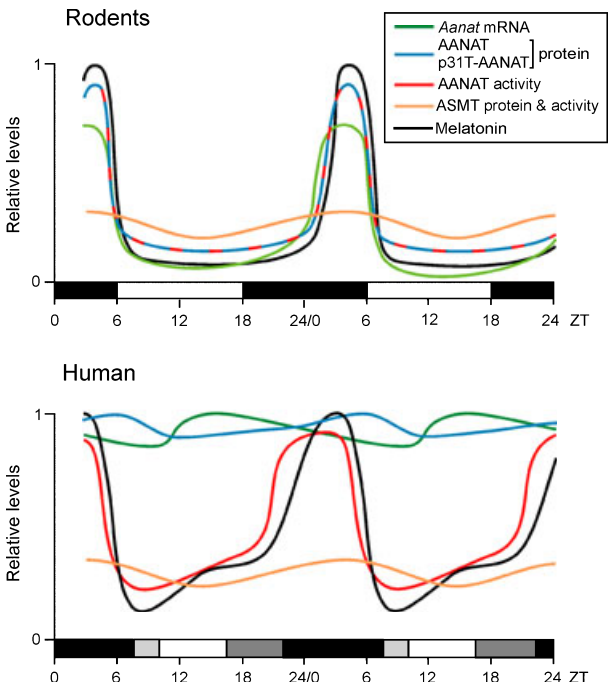

Fig. 4. Dynamics in melatonin synthesis, compared between rodents and human. The schematic patterns show relative values of relevant rhythmic and nonrhythmic parameters of melatonin synthesis, as deciphered in the rodent pineal gland and in human autoptic pineal tissue. Depicted are *Aanat* mRNA, arylalkylamine *N*-acetyltransferase (AANAT) and p31T-AANAT protein content, AANAT and acetylserotonin *O*-methyltransferase activity, and melatonin content over a 24-hr light/dark cycle (ZT, Zeitgeber time). Nighttime: black bar; dawn: light gray bar; day: white bar; dusk: dark gray bar. Note that *Aanat* transcription is determining dynamics in melatonin synthesis only in rodents. For better visualization, relative values are double-plotted against asserted Zeitgeber time (ZT). Data are deduced from relevant literature [87, 96].

increased *Aanat* transcription is regulated by activating (for example, phosphoCREB, AP1) and inhibitory (for example, ICER, DREAM) transcription factors [8, 89, 95, 98, 99].

In rat pinealocytes, post-translational mechanisms were shown to participate in signalling events. At the beginning of the night, the up-regulation of *Aanat* transcription by the activating transcription factor cAMP-response element binding protein seems to be flanked by a proteasomal proteolysis of inhibitory transcription factor(s) [8, 89, 100, 101]. Thereby, the AANAT protein seems to be shielded from destruction by a highly conserved NE-induced, cAMP-dependent, protein kinase A (PKA)-mediated phosphorylation mechanism, involving the 14-3-3 delta/epsilon scaffold protein. It is only at the end of the night that proteasomal proteolysis can effectively and rapidly degrade AANAT [102].

In addition to the above-mentioned transcriptional mechanisms, recent studies have demonstrated that chromatin remodelling is a naturally occurring event, also in the rat pineal gland [103–105]. Studies showed that histone phosphorylation and acetylation are a cAMP-inducible phenomenon that is restricted to nighttime, thus exactly coinciding with the demand of an enhanced gene transcrip-

Table 1. Dynamics in the pineal gland of various parameters in different vertebrates

	AANAT			Asmt					
	Aanat	Protein	Activity	*Asmt*	Protein	Activity	Melatonin	Circadian clock	Photo-sensitive
Lower vertebrates									
Sauropsids	∿	∿	∿	n.i	n.i	n.i	∿	∿	Yes
Chicken	∿	∿	∿	∿	–	n.i	∿	∿	Yes
Mammals									
Mouse/rat	∿	∿	∿	∿		∿	∿	–	No
Octodon degus	–	n.i.	n.i.	n.i.	n.i.	n.i.	∿	n.i.	No
Sheep	–	n.i.	∿	–	–	–	∿	–	No
Monkey	–	–	∿	–	n.i.	–	∿	–	No
Human	–	–	∿	–	–	–	∿	–	No

AANAT, arylalkylamine *N*-acetyltransferase. Comparison of diurnal dynamics for important pineal parameters in different vertebrate species. ∿ indicates a day/night rhythm; –indicates a constitutive pattern. For details and references, see text. Abbreviation: n.i., not investigated.

tion. Moreover, remodelling of chromatin participates in the transcriptional regulation of a selective number of rhythmically expressed genes, notably the *Aanat*, *c-fos* and *Icer* genes [100, 103–105]. These findings add a new level of regulation within signalling events in the rodent pineal gland, as the inducible histone-dependent changes in DNA conformation control the accessibility of gene loci to the binding of transcription factors, thereby preceding transcription itself.

Sheep and monkey

In the sheep pineal gland, *Aanat* mRNA levels do not show differences between day and night (Table 1)[94, 106, 107], despite a marked circadian change in AANAT activity [94]. Moreover, in sheep, constitutive *Aanat* levels cannot be further increased by stimulation of the cAMP signalling pathway with forskolin [108]. These observations indicate that in the rhythmic melatonin synthesis in the sheep pineal gland, the transcriptional control is very much attenuated, when compared with rodents (see section: Melatonin synthesis in the vertebrate pineal gland: Rodents).

Similar to sheep, no differences in *Aanat* or *Asmt* mRNA levels between day and night could be detected in the primate *Macaca mulatta*, while AANAT activity and AANAT protein were tenfold higher at nighttime, when compared with daytime (Table 1)[109]. Experiments carried out on ovine [94, 108] and bovine pinealocytes [110, 111] demonstrated that constitutively elevated *Aanat* mRNA levels lead to a constant production of AANAT protein, which seems to be instantly degraded during daytime via proteasomal proteolysis [94, 102]. At least in sheep, it was shown that at nighttime, however, the accumulation of cAMP in pinealocytes, induced by the increased NE release from the sympathetic innervation of the gland, induces AANAT activity, leading to the nocturnal melatonin surge [94, 102]. This biochemical switch from a destructive targeting of the AANAT by the proteasome towards maintenance in the activity of the penultimate enzyme for melatonin synthesis was shown to occur via a cAMP-dependent and PKA-mediated formation of a complex containing AANAT and 14-3-3 protein [112, 113]. In analogy to these observations made in ungulates and in the monkey, it was so far assumed that dynamics in melatonin synthesis are similarly regulated in the human pineal gland [114, 115].

Human

The measurement of melatonin in human body fluids (serum, saliva) or one of the major melatonin metabolites, 6-sulfatoxymelatonin, in urine samples [93, 116] revealed a rapid adjustment of the hormone's rhythm to ambient lighting conditions [117, 118]. The rhythm in circulating melatonin levels in man is of truly circadian nature, as it persists even in the absence of a light/dark cycle [39, 93, 119, 120].

The DLMO in the human matches temporally with ex vivo observations made in sheep and monkey [91, 93, 116, 121, 122], where it was shown that this dynamics is accomplished by a rapid increase in AANAT activity, which implies already at that time-of-the day the presence of AANAT protein and thus of *Aanat* mRNA (Fig. 4, Table 1). This dynamic in onset of melatonin synthesis and its fast decline only with light at dawn are in strong contrast to the situation in rodents, where the onset of melatonin synthesis is delayed after dusk for several hours and where hormone production declines in anticipation of dawn [5, 88, 123–125].

While species-specific differences in the on- and offset of melatonin synthesis (and thus in its regulation) are clearly evident, a very rapid decline in nocturnally elevated hormone synthesis was observed in all vertebrates upon application of even low-light intensities, indicating the involvement of very fast acting biochemical processes, like protein modifications [112]. Notably, this regulatory capacity of light to suppress melatonin synthesis is also effective in man [126–128].

The development of accurate and sensitive assays to measure melatonin in plasma and saliva has contributed major advances in our understanding of the temporal fine structure of the melatonin rhythm, at the same time providing a phenomenological basis to predict the signalling dynamics on the molecular level present in the human pineal gland.

It should be noted here that in the human brain, the pineal gland is not the only source of melatonin. The lateral eyes are able to rhythmically synthesize small amounts of

melatonin [129]. This is not surprising from a phylogenetic standpoint, as the eyes share their diencephalic origin with the pineal gland [8, 62]. Animal studies showed that melatonin-secreting photoreceptors and dopamine-secreting amacrine and interplexiform cells form a cellular feedback loop, regulating circadian retinal physiology [130]. Retinal rhythms are driven by a hidden but detectable circadian clock [131, 132], possibly even in the human eye [133]. There is convincing evidence that retinal melatonin only acts locally and does not reach other brain structures [133].

In the human, low serum melatonin concentration during daytime (<20 pg/mL plasma) rises up to 100 pg/mL plasma during the night [116, 119], with rhythmic synthesis appearing between the second and third month of life [134]. The lack of a functional circadian system during early foetal life is overcome by the communication of circadian timing cues by the 'sighted' mother. The rhythmic maternal melatonin signal circulates in the blood stream, readily passes the placental barrier and serves in utero the need to synchronize the foetal clock(s). Animal studies showed that maternal/foetal communication continues postnatally until full maturation of the circadian system, as maternal melatonin is transmitted to the newborn via breastfeeding and thus efficiently fulfils the role as an important daily Zeitgeber to communicate the duration of nighttime [135–137]. A pronounced daily melatonin rhythm was also detected in human milk [138], with high levels during the night and undetectable levels during the day. As a consequence, a disturbed maternal melatonin synthesis or early weaning may lead to an insufficient synchronization of neonates with their mothers and therefore with the environmental 24-hr day. This observation underlines the importance of an intact and rhythmic melatonin generating system in man that is only preserved when the central clock in the SCN is intact.

However, in highly industrialized societies, the artificial lighting leads to a major disturbance of the circadian rhythm generating system in humans in general ('social jetlag') [78, 139, 140] and in nocturnal melatonin levels in particular [39, 47]. Night work or shift work disrupts the normal sleep/wake pattern, leads to a significant reduction in urinary 6-sulfatoxymelatonin levels and correlates with increased levels of bioavailable oestradiol that itself seems to correlate with a higher risk to suffer from breast cancer [141]. This link is further supported by in vitro experiments that show the antiproliferative potency of melatonin in oestrogen-sensitive breast cancer cells [142–144]. As a result, the restoration of the melatonin rhythm in 'socially jetlagged' people became an emergent therapeutic goal. However, the apparent lack of knowledge regarding the molecular details on the regulation and maintenance of the melatonin signal in humans impedes on the development of preventive or curative treatment strategies, which can only be overcome by investigation made in postmortem tissues.

The general validity of using autoptic brain material, with analysis afflicted by variations in the postmortem intervals (PMIs) and an unknown premortem lifestyle, has been extensively demonstrated earlier [145–147]. In these studies, the quantitative relation of different mRNAs with each other was remarkably preserved postmortem, although levels of individual mRNAs were highly variable between subjects. Thus, shortlisted data cleared from influential effects like varying length in PMIs, in gender and in age, allowed testing results for significant differences between time-matched groups across a 24-hr day/night period [145–147].

The principal feasibility of using autoptic pineal tissue for analysis of premortem diurnal dynamics was shown in an early case study [51], where residual AANAT and ASMT enzyme activities were detected. Subsequently, a principal decline in day/night differences in AANAT amplitude with age and with the development of Alzheimer's disease (AD) was shown (Fig. 5)[52, 148]. Based on these promising earlier publications, recent studies investigated autoptic pineal tissue for remnant molecular signatures, related to the generation of rhythmic melatonin synthesis in man [87, 149]. Studies demonstrated that intact mRNA and protein

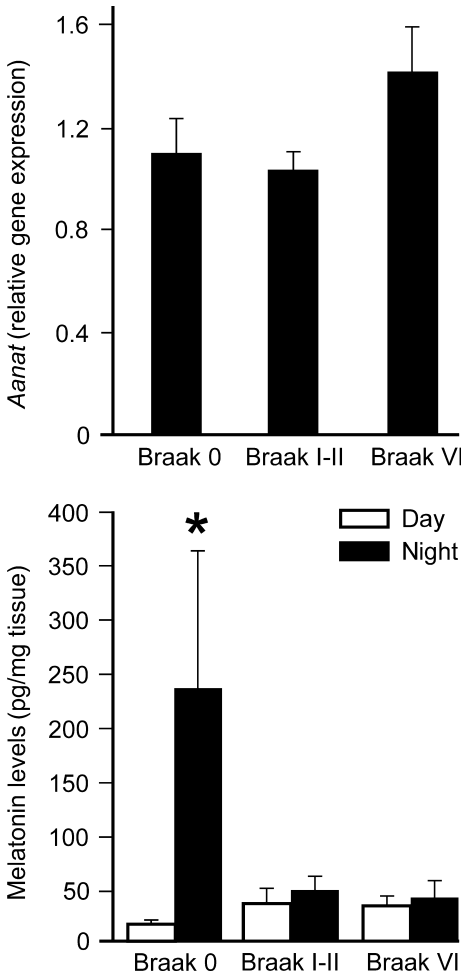

Fig. 5. Decline of *Aanat* mRNA and melatonin levels with progressive stages of Alzheimer's disease (AD). *Aanat* gene expression (upper graph) and changes in day/night pineal melatonin levels (lower ghraph) in relation to AD progression (indicated as different Braak stages [265]). Values are derived from analyses of autoptic pineal tissue (modified with permission from [52]). Note that the decline in nocturnal melatonin synthesis occurs already during Braak stages I-II and is not accompanied by a simultaneous decline in *Aanat* expression.

could be acquired from pineal tissue samples provided from forensic medicine autopsies.

Molecular details of human melatonin synthesis

Initially, a linear and almost parallel degradation process with time was demonstrated for different mRNA species and various proteins in the human pineal gland [87, 149, 150]. Pineal tissues from more than 100 specimens were analysed in parallel for *Aanat* and *Asmt* mRNA content, for AANAT and ASMT enzyme activities and for melatonin levels. Thereby, a remnant silhouette in biochemical markers for the premortem rhythmic neuroendocrine activity in the human pineal gland was detected. Melatonin content showed a clear daytime-dependent difference, with elevated levels restricted to nighttime, matching diurnal hormone profiles assayed in healthy men, using noninvasive or minimally invasive techniques [93]. In contrast, neither *Aanat* or *Asmt* mRNA levels nor ASMT activity showed significant day/night differences [52, 87].

To exclude any possible unknown contributing variables originating from the diversity of anamnestic parameters, the investigated specimens were grouped based on gender, age and PMI to confirm the absence of *Aanat* and *Asmt* rhythmicity in the human pineal gland [87, 150]. Notably, regardless of subgroup characteristics, the absence of any diurnal rhythm in human *Aanat* and *Asmt* rhythmicity matched pineal data obtained in the whole cohort [87]. These results strengthened the assumption that post-transcriptional mechanisms are crucial for the generation of rhythmic melatonin synthesis in the human pineal gland.

Interestingly, dynamics in pineal parameters as seen in autoptic pineal tissue were likewise observed in fresh sheep pineal tissue [106, 151], supporting the indications for parallel biochemical mechanisms that drive the nocturnal melatonin signal in ungulates and primates. In addition, in human autoptic and fresh sheep tissue sections, an identical intracellular localization of the AANAT protein was found (Fig. 6; [151]). In both species, AANAT immunoreaction was localized to the cytoplasm close to, or in the perinuclear region, and also extensively within the network of cellular processes of pinealocytes (Fig. 6) and is thus unlikely caused by decomposing of the autoptic human tissue. It may be assumed that the NE-driven melatonin biosynthesis machinery in human and sheep pinealocytes is compartmentalized in a 'melatoninosome'. As such 'melatoninosome'-containing processes were principally described earlier [152, 153] and were not observed in AANAT- and ASMT-cotransfected HeLa cells [151], it may be a neuron-specific or even a pinealocyte-specific subcellular structure.

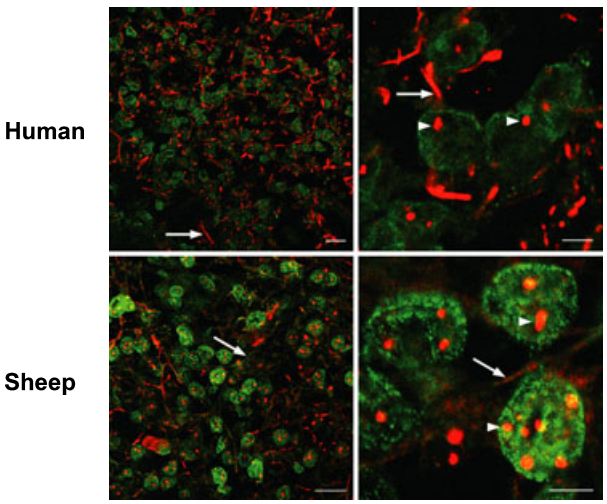

Fig. 6. Arylalkylamine *N*-acetyltransferase (AANAT) protein in the human and sheep pineal gland. Immunoreaction of AANAT (*red*) and of nuclear pore complex protein (*green*), visualized in 5-μm cryostat-cut sections of autoptic human pineal tissue (upper panels), or in sheep pineal sections (lower panels), using confocal laser scanning microscopy. Note the similar AANAT immunoreaction in human and sheep pinealocytes in the nuclei (arrowheads) and in tubular cytoplasmatic processes (arrows). Scale bars: 20 μm in left column, 5 μm in right column (modified with permission from [151]).

Phenomenologically, in sheep and human, neither the intensity of the AANAT immunoreaction, as detected with a specific antibody against the phosphorylated T31-AANAT (p31T-AANAT) nor its intracellular localization changed with the time-of-day [151]. This provides evidence against the existing hypothesis that only proteasomal degradation of AANAT during daytime determines rhythmic enzyme abundance [115]. Moreover, immunofluorescence double labelling experiments and coimmunoprecipitation studies supported the colocalization of AANAT, ASMT and the 14-3-3 protein in the subcellular compartments of human pinealocytes. Thereby, ASMT was shown to be associated with the AANAT/14-3-3 complex, independent of the time-of-day [151]. These novel observations indicate that the stabilizing complex is constitutively present and that the earlier noted assumption of a dephosphorylation of the AANAT at T31 during daytime does not account for rhythmic AANAT activity in the human pineal gland [151]. In this context, the possible importance of other so far sparsely investigated phosphorylation sites of the AANAT protein, e.g. at residue S205, requires further investigation.

When analysing the remnant silhouette of melatonin synthesis in autoptic human pineal tissue, it occurred that significantly elevated AANAT activity levels during dusk were unexpectedly not paralleled by simultaneous elevation in melatonin content, but hormone concentrations increased some 4 hr later only (Fig. 4; [87]). One explanation for this delay may be the existence of an intermediate step with a rate-limiting potency for the conversion of the enzymatic AANAT product, *N*-acetylserotonin, into melatonin. As this biochemical step is achieved by ASMT, it is of interest that in the rat and hamster pineal gland, of a vast excess of *N*-acetylserotonin is not mirrored in a proportional increase in melatonin synthesis [60, 61]. *N*-acetylserotonin is optimally synthesized inside pinealocytes at a pH of 6.8, while its optimal methylation by ASMT requires a pH of 7.9 [154]. The functional meaning of this difference in pH optimum and the cellular resolution of these biochemical demands remain to be elucidated. It can be envisioned that intracellular translocation of the *N*-acetylserotonin may account for the observed temporal gap between the elevated AANAT activity and the increase in melatonin levels in human pineal tissue.

In summary, the assumption that a temporally gated proteasomal proteolysis of AANAT during daytime drives rhythmic melatonin synthesis in the human pineal gland has to be revised. Rather, on the basis of a constitutive AANAT expression, AANAT activity seems to be regulated inside a 'melatoninosome' by protein–protein interactions with the 14-3-3 protein, ASMT and S-antigen [151].

The reasons for the development of different regulatory mechanisms for melatonin synthesis in rodents and ungulates/primates remain speculative [8, 106], but may be related to an animal's lifestyle. Most rodents live in burrows and are active during nighttime, and some of them do not see light for months during their hibernation period. In these species, the stable nature of the transcriptionally controlled melatonin signal is a potent and reliable Zeitgeber to time remote body clocks. Under the conditions of nighttime activity and daytime rest, the anticipation for the break of day, and thus the timing of activity and food intake, and the escape from daytime predators, is crucial for survival. Thereby, the melatonin signal seems to gate and control circadian behavioural rhythms in rodents through adjustment of the circadian oscillator in the SCN [155–160]. In contrast, most ungulates and primates do not hibernate; they live in unsheltered environments, are exposed during their activity period to the daily lighting regimen and interpret dusk at the start of their rest period. The activity gating signal of sunrise and sunset may have weakened the need for melatonin to adjust the SCN clock rhythms, and this may have diminished the necessity of a stringent transcriptional control of the melatonin rhythms. The lifestyle of most ruminants and primates also implies that the anticipation of dawn is less important when compared with rodents. Indeed, in these species, the melatonin signal is only weakly coupled to the circadian clockwork entraining pathways [39, 161–164], indicating that the seasonal variations of the melatonin surge gained a superior importance and control seasonal physiology of reproduction and metabolism [68, 106, 165].

Physiological role of human melatonin synthesis

The temporal structure of the melatonin synthesis rate in man is very reproducible, on a daily basis, in a given subject (see for an example: Fig. 9). However, like a 'hormonal fingerprint', there are specific interindividual differences and notably heritable patterns of the temporal design of the nocturnal melatonin surge in man [39, 166, 167]. Moreover, nocturnally elevated melatonin levels change in amplitude and duration during lifetime, as investigated using plasma, serum, urine excretion of 6-sulphatoxymelatonin and saliva. Most studies reported a steady decline of 20–80% of prepubertal melatonin levels in the progressed age of 55 yr and beyond, often accompanied by an increase in daytime hormone levels [168–176]. While the huge decrease in hormone levels during childhood and adolescence (Fig. 7) is caused by the dramatic extension of body size [172], the moderate decline at older ages has its reasons in ageing processes, possibly including degenerative dynamics in pineal physiology and/or in SCN clockwork integrity [177, 178] (see section: Disturbed melatonin synthesis, causes and curative countermeasures). The stability of an individual melatonin profile allows alike the diagnosis of dysfunction in the photoneuroendocrine/retina/SCN/pineal axis, such as circadian misalignments, appearing as a consequence of ageing or of pathophysiological states, inevitably accompanied by an altered melatonin profile. Indeed, analyses of nocturnal melatonin values in autoptic pineal glands showed a disappearance of significant day/night differences with age [148].

When compared with melatonin measurement in body fluids, data derived from autoptic pineal tissue have the advantage to bypass possible altered metabolic processes

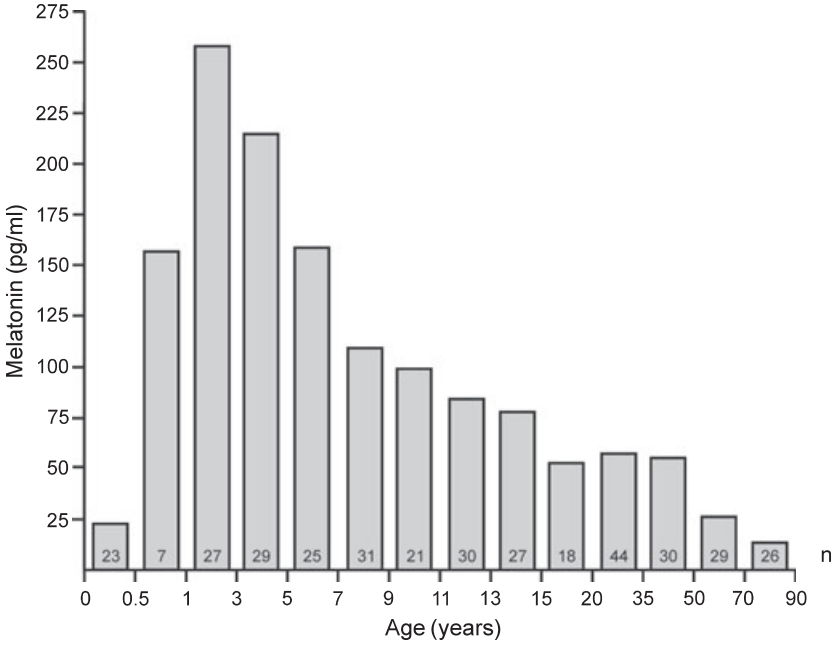

Fig. 7. Age-dependent decline in melatonin synthesis. Analysis of nocturnal serum melatonin concentrations, as analysed from 367 subjects, with number of individuals indicated inside columns (modified with permission from [172]).

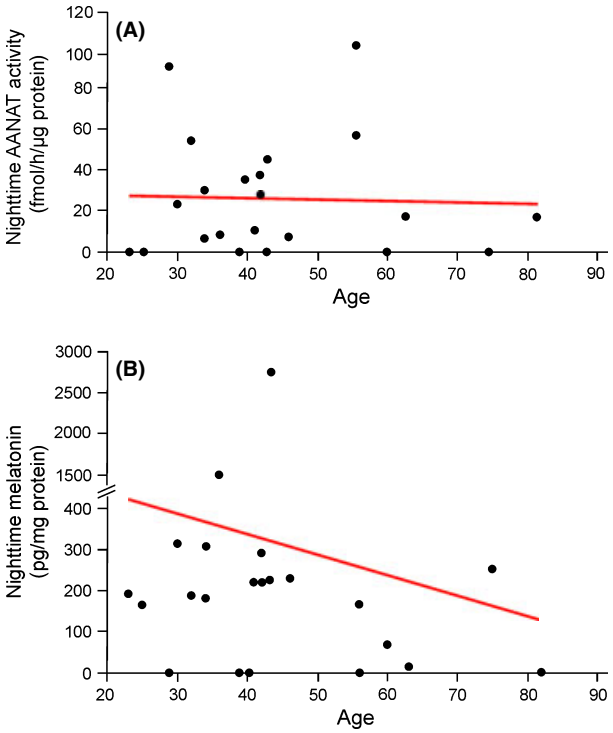

Fig. 8. Age-dependency in human melatonin synthesis. Correlation of nocturnally elevated levels in arylalkylamine *N*-acetyltransferase (AANAT) activity (A) and melatonin levels (B) with age in human pineal glands (n = 22). Regression lines have an age-dependent negative slope (AANAT: −0.043; melatonin: −4.87). Note the interrupted scaling of the ordinate in B. Note also that a significant day/night difference in AANAT activity and melatonin levels in general were observed earlier in the same specimen, which was shown to be independent of age [87].

outside the pineal gland, but they endow the disadvantage that degradation processes because of PMIs have to be taken into consideration. We conducted recently a post-analysis of 22 pineal glands of subjects that died exclusively during nighttime (Fig. 8). Obtained data substantiate the earlier reported decline in the amplitude of the melatonin rhythm in blood with increasing age of subjects (slope of the regression line: −4.87), confirming also observations made in serum [97]. Even after exclusion of individuals with melatonin values below detection limit (n = 5) and with very high melatonin values (n = 3), respectively, a negative trend with age was evident (slope of the regression lines: −2.02)(Fig. 8B). AANAT activity declined with age, showing a negative regression line of moderate slope (Fig. 8A; slope of the regression line: −0.043; when values below detection excluded: −0.23), which resembles earlier reports on an absent age- or dementia-related decrease in enzyme activity [52]. From these analyses, it can be concluded that the rather small decline in AANAT activity in the human pineal gland with age is most likely not the only reason for the diminished melatonin dynamics in the elderly. Rather, an increasing age may affect ASMT or may compromise the post-translational processing of enzymes involved in melatonin synthesis.

The alteration in melatonin synthesis in elderly people was also suggested to be related to an ageing clockwork, e.g. a deterioration of neuronal function in SCN cells [178–180], a weakening in the retinal entrainment pathway by light through the retino-hypothalamic pathway [181], or to a reduced strength in nonphotic time cues like social interaction that serve as additional Zeitgebers for the human circadian system [139]. Moreover, an age-dependent decline in vasopressin and in MT gene expression in the human SCN has been reported [182, 183]. Regarding MT, its expression is noteworthy that it is common for G-protein-coupled receptors to be down-regulated upon a lengthened absence of ligand binding [184]. Investigations using human material have shown that the decrease in melatonin amplitude with age may be coupled to a diminished presence of adrenergic receptors on membranes of human pinealocytes [52]. In return, the altered noradrenergic regulation of the melatonin rhythm with age may be used to mirror an onset of neuronal degenerative processes.

Disturbed melatonin synthesis, causes and curative countermeasures

To assess dynamics in human melatonin levels, the DLMO is a reliable index marker at dusk and has proven to be one of the best available parameters to monitor the functional state of the endogenous circadian clock in the SCN. The temporal structure of the DLMO is very reproducible in a given subject over days [119, 166]. Therefore, the DLMO has been playing an increased role in the diagnosis of circadian rhythm disorders [42, 43, 91], and to preset the schedule for timed bright light exposure and/or for low-dose melatonin administrations for therapeutic purposes [40, 42, 43, 93, 185], as the difference in time between DLMO and mid-sleep (phase-angle difference, PAD) can be used as a marker for internal circadian alignment (or misalignment) between the SCN clock and the rhythm in melatonin synthesis. The PAD may also be used to differentiate individuals who experience permanent (for example by shift work schedule) or acute (for example because of trans-meridian flights) phase advances (large PAD = long interval) from those who experience a phase-delayed internal clock (short PAD = short interval). To provide a corrective phase delay, melatonin or newly designed melatonin agonists that act as potent chronobiotics in adults have to be administered in the morning, while a phase advance is achieved by melatonin treatment in the afternoon/evening [40, 92, 93, 162]. Already, treatments with physiological concentrations of melatonin were shown to be particularly helpful in an effort to ease or even counteract the discomfort of blind people that lack light entrainment [39, 91–93, 120, 162, 186] and in institutionalized patients suffering from AD [182]. There is also evidence for a positive effect of melatonin to ease circadian rhythm sleep disorders [42, 43, 185, 187]. In general, the mechanisms behind the curative effects of melatonin are believed to be based on the interaction of the hormone with the human circadian clockwork through binding to melatonin receptors expressed by SCN cells [38].

Reports on the benefits of reconstructed melatonin rhythms in the elderly and in elderly demented patients are controversial [42, 43, 188, 189]. Clearly, melatonin

improves some cognitive and noncognitive symptoms of dementia, when given in combination with bright light therapy [190]; however, these beneficial effects may be explained by melatonin positively interfering with the early decline in core body temperature in the elderly, appearing at early evening [45, 191, 192]. Melatonin could thereby improve sleep induction and sleep maintenance through gating the rest period of the brain during nighttime by its soporific capacity [42, 43, 161, 193, 194]. An (possibly melatonin-mediated) improvement in sleep quality, as reported to be beneficial for memory consolidation and re-consolidation in healthy human subjects [195, 196], may also improve the health state of elderly demented patients.

There is growing evidence that the aberrant dynamics in melatonin synthesis is more than a consequence of a comprehensive rhythm disorder found in the elderly demented AD patients, in patients with the Smith–Magenis syndrome (SMS) or in those suffering from autism spectrum disorder (ASD) and delayed/advanced sleep phase syndrome (DSPS/ASPS). It rather seems that a dysfunction in signalling events in melatonin-producing pinealocytes may be related to the manifestation of the above-mentioned pathological phenotypes. A more appropriate curative intervention according to the chronopathological state of a given subject requires a comprehensive knowledge of the molecular details behind the melatonin rhythm in man (see section Human), which will inevitably foster the treatment of internal circadian misalignment by pharmacological intervention.

Alzheimer's disease.

More than 90% of people older than 65 yr of age complain about circadian rhythm disorders [197]. They display general clinical symptoms of an affected chronotype found in elderly demented patients (sundowning, i.e. a phase delay in the acrophase of the body temperature rhythm; fragmented sleep/wake pattern), which largely emerges in patients with AD [174, 189]. In particular, the decline in amplitude of the nocturnal melatonin surge with age (see Figs 5, 7 and 8) is reinforced with increasing mental impairment [169] and irregularities in hormone secretion, known to be predominantly severe in patients with AD [174, 189, 198, 199]. The AD-related melatonin secretion rhythm disorder may result from enhanced ageing of the SCN clock. However, there have also been reports on AD-related alterations in human pinealocytes, in addition to the described age-dependent changes. For example, the well-documented day/night rhythm in β_1-adrenergic receptor expression in mammalian pinealocytes [200–202] declines in humans during early preclinical stages of AD (Braak stages I–II [265]), when compared with age-matched healthy subjects [52, 182, 203]. This decline in β_1-adrenergic receptor mRNA may result in lower receptor expression and lead to a reduced adrenergic stimulation of melatonin synthesis during nighttime and thus may be used as an early indicator to determine the stage of AD. While this causative link may hold true, it has to act distally of *Aanat* expression, as mRNA levels remain unaffected wth progressing AD stages (Fig. 5)[52]. In addition to the dysfunction of noradrenergic signalling, an age-dependent depletion of the local serotonin stores, serving as the substrate for melatonin synthesis, may be causative for the loss of a rhythm in pineal hormone synthesis and secretion already during preclinical stage of AD.

The finding that melatonin inhibits amyloid deposition in an AD mouse model [204] urges clinical investigations into the potential beneficial effect of a reconstructed melatonin synthesis by pharmacological interventions. While the therapeutic supplementation of the pineal hormone surely eases the painful AD-related impairment of social integration, it may be assumed that this intervention also slows down AD development. Indeed, in institutionalized patients with AD, suffering from rest/activity disruption, the administration of melatonin in moderate pharmacological doses (5 mg/day) in parallel with light therapy increased day wake time and activity levels and strengthened the rest/activity rhythm (Fig. 9)[185]. Notably, in this study, light treatment alone was ineffective to cure AD-related chronobiological dysfunctions, like disrupted nighttime sleep, daytime wakefulness and the general instability of the rest/activity rhythm.

Taken together, there are strong indications that a harming of the integrity of the SCN–pineal connection with progression of AD is causally related to circadian rhythm disorders in these patients. A reconstruction of a

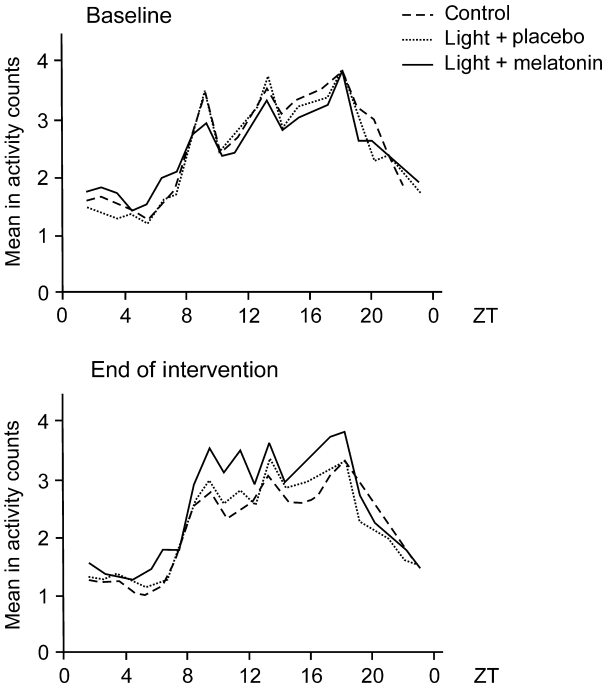

Fig. 9. Effect of melatonin and bright-light cotreatment in patients with Alzheimer's disease (AD). Hourly registered activity counts in a group of institutionalized patients with AD at baseline (upper graph) and at the end of the 10-wk intervention period (lower graph), with subjects treated either with bright light (> 2.500 lux) in the morning or with bright light together with melatonin (5 mg)(modified with permission from [185]). Note the consolidation of daytime activity in the treated groups at the end of intervention when compared with controls. Note also the higher improvement in consolidation when melatonin was given in addition to bright light, when compared with the group receiving bright light treated alone.

proper melatonin signal may not only be beneficial for circadian rhythm disorders in general, but in particular to decelerate the progress of AD.

Smith–Magenis syndrome.

Smith–Magenis syndrome is a severe neurodevelopmental disorder, characterized by a *de novo* microdeletion on chromosome 17 at position p11.2 (prevalence is one in every 25.000 births). Patients with SMS suffer among other symptoms like cranio-facial phenotype and general mental retardation, sleep disturbances and maladaptive daytime behaviour [205]. The latter is ascribed to the genetically determined paradoxical inverted melatonin synthesis, with elevated hormone levels occurring during daytime. These erratic kinetics in melatonin secretion cannot be considered as a generalized chronobiological disorder, as circadian rhythms in cortisol, prolactin and growth hormones are normal (Fig. 10) [206]. While the quest for the possible molecular cause for SMS-related melatonin deregulation showed close physical chromosomal mapping of the SMS locus (17p11.2) to the *Per1* gene (17p12), the exact mechanism is still unknown.

The above-mentioned findings suggest that the abnormal circadian melatonin rhythms and altered sleep patterns associated with SMS could be downstream of the aberrations in the production, secretion, distribution or metabolism of melatonin. The quantitatively normal, but temporally inverted melatonin secretion in SMS may be disease-evoked by a modified adrenergic receptor expression in pinealocytes (similar to AD), an altered monoamine metabolism or an aberrant retinal-entraining pathway. The latter could imply an abolished conversion of the nocturnally decreased sympathetic activity into an activating signal for pineal function, as it occurs in all diurnally active mammals [7]. Alternatively, a re-uptake of NE by sympathetic nerve endings during daytime from the vicinity of pinealocytes [207] may be missing and thus allow the (stress-) increased sympathetic tone to stimulate melatonin synthesis at an inappropriate time, namely during daytime. Interestingly, it was shown in a case study that in a 4-yr-old patient with SMS, the treatment with β_1-adrenergic antagonist in the morning (to inhibit aberrant increases in melatonin synthesis) and with melatonin in the evening (to mimick the nocturnal melatonin peak) improved nocturnal sleep quality [208].

Autism spectrum disorder.

In patients with ASD (characterized by major impairments of communication skills and social behaviour; prevalence six in every 1.000 births), alterations in the sleep/wake rhythm and low melatonin levels throughout day- and nighttime are observed (Fig. 11, [209, 210] and references therein). Notably, ASD is highly correlated with the deletion of the *Asmt* gene (located on the pseudoautosomal region of the human X (Xp22.3) and Y (Yp11.3) chromosome [211,

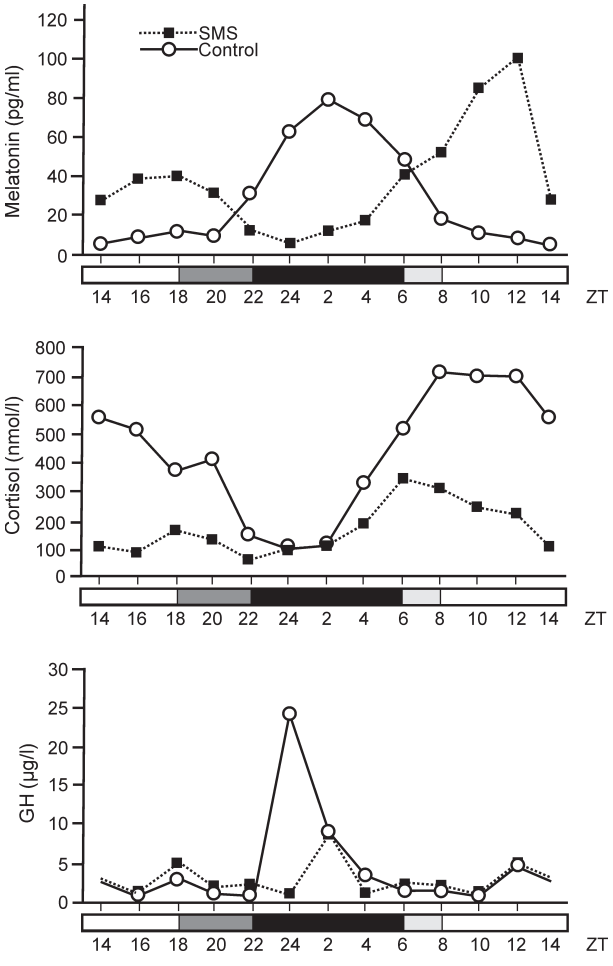

Fig. 10. Melatonin rhythm in patients with Smith–Magenis syndrome (SMS). Circadian variations of serum melatonin levels (upper graph), cortisol (middle graph) and growth hormone (GH; lower graph) in a representative SMS patient and a control subject (modified with permission from [206]). The black bar indicates night; dusk and dawn are indicated in grey. Note the parallel rhythms in diurnal cortisol and GH secretion, when compared with the inverted day/night melatonin rhythm between the two individuals. ZT, Zeitgeber time.

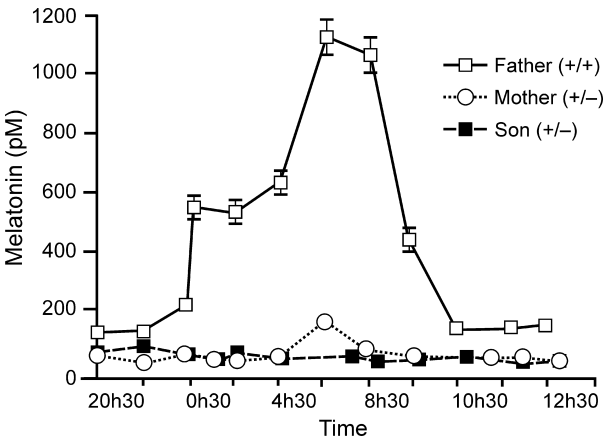

Fig. 11. Diurnal melatonin profile in autism spectrum disorder (ASD)-affected individuals. Shown are members of an ASD-affected family (modified with permission from [209]). Note the normal profile in diurnal melatonin synthesis in the father (+/+), who is no patients with ASD and has no acetylserotonin *O*-methyltransferase (ASMT) mutation, and the absent rhythm in melatonin synthesis in the heterozygous ASMT-mutated mother and son (+/–).

212]), which encodes the final enzyme in melatonin synthesis [6, 7]. A recent genetic investigation into ASD-affected families showed that *Asmt* gene polymorphisms seem to lower *Asmt* transcript levels and/or ASMT protein content [209]. While this study cannot consider low ASMT activity as the direct cause of autism, it shows that it is a susceptibility factor. The analysis adds to the known modulating roles of melatonin in cognitive processes [213] and indicates that the pharmacological stimulation of melatonin synthesis during nighttime and/or application of melatonin as a therapeutic compound may be beneficial in counteracting the consequences of ASD in man. Indeed, a recent report shows an improvement in the ASD-affected sleep pattern by morning application of a β_1-adrenergic antagonist and an evening dose of melatonin [208].

As a consequence of ASD-related lowered ASMT activity, the ASMT substrate, *N*-acetyl-serotonin, accumulates in these patients. *N*-acetyl-serotonin is a 'natural pain killer' by inhibiting the production of tetrahydrobioptin [214]. Interestingly, reduced CSF concentrations of tetrahydrobiopterin and reduced peripheral pain perception have been reported in autistic children [215] and in patients with ASD [216], respectively. This biochemical link asks for a therapeutic concept to rebuild the nocturnal melatonin surge to counteract the reduced peripheral pain perception in patients with ASD.

Sleep phase syndromes (SPS).

The DSPS is a circadian rhythm sleep disorder, commonly affecting adolescents, with sleep-onset insomnia accompanied by difficulties in arising. While psychosocial and environmental factors have been suggested to be causally related to DSPS, it was recently demonstrated that both a tandem-repeat polymorphism and a single-nucleotide polymorphism in the *Per3* gene were strongly correlated with DSPS [217, 218]. In these patients, the DLMO is also delayed, when compared with normal subjects [219]. Notably, the allelic positivity of the single-nucleotide polymorphism at position 619 (G- > A) of the *Aanat* gene was significantly higher in patients with DSPS, when compared with healthy controls [220]. This finding suggests that *Aanat* could be a susceptibility gene, targeted in the DSPS circadian rhythm sleep disorder. Thus, pharmacological interventions targeting the final biochemical steps in melatonin synthesis may counteract the DSPS-associated sleep disturbances by restoration of the clinically conspicuous daily pattern of melatonin in man.

Patients suffering from advanced SPS (ASPS) have a stable sleep schedule that is, however, several hours advanced when compared with the conventional sleep-onset time, a phase shift that can easily be monitored by analysis of the DLMO. Recent studies have detected that a pedigree to an altered structure and/or processing of the *Per2* gene product exists for ASPS. Such single-nucleotide polymorphism affects transcriptional and post-transcriptional regulation of this clock gene and thus circadian rhythm generation in the SCN [221]. While systematic reports on the possible beneficial effect of a phase adjustment of the melatonin rhythm in ASPS-affected patients are missing, a rational for administration after awakening is indicated. In contrast to DSPS, the latter observations point out that the affected pineal signalling in ASPS is secondary to the affected central clockwork in the SCN.

Clock gene expression in the vertebrate pineal gland

A self-sustained circadian rhythm generation is a hallmark to all biological systems for the anticipatory adaptation to environmental changes within the 24-hr solar day. Intrinsic clocks are utilized by the simplest to the most complex animals [222].

In lower vertebrates, oscillating clockworks can be found in almost every somatic cell, including the directly photosensitive pineal gland. Circadian rhythms are driven by a distinct number of clock genes, namely members of the *Period* (*Per*) and *Cryptochrome* (*Cry*) families, of *Bmal1*, and presumably of *Clock* or *Npas2*, respectively, and their corresponding protein products (PER, CRY, BMAL1, CLOCK/NPAS2). In interlocked feedback loops, clock gene proteins form transcriptionally active complexes that interact with their own genes and thereby provide the basis for a perpetual function of the clockwork and for a rhythmic regulation of clock-controlled output genes [222–224].

In mammals, clock genes are expressed in many tissues, but they are in particular the molecular elements, driving the endogenously sustained circadian rhythm in the SCN-based master oscillator. Despite this evolutionary change from photosensitive and clockwork-endowing tissues in lower vertebrates into a hierarchical organized circadian system in mammals [223, 224], cycling clock gene expressions can be detected in almost all 'light-unresponsive' extra-SCN mammalian tissues investigated [225–227], including the pineal gland [79, 150, 224–226, 228, 229], with the function of clock genes and their proteins possibly being reduced to their potency to act as time-measuring transcription factors.

To improve our understanding of the mechanism(s) of rhythmic melatonin synthesis, a comparative analysis of clock gene expression (Table 2) and their regulation in different vertebrate species is necessary. Consequently, the putative role of these time-measuring molecules in the human pineal gland and their possible role in the generation of the nocturnal melatonin surge can be elucidated.

The nonmammalian pineal gland

A direct participation of clock gene products in pineal function has so far only been shown in the pineal gland of some fish and chicken. In the zebrafish, autonomous rhythms even persist in vitro in the majority, if not in all tissues. These rhythms can be entrained by photoperiodic cues and are based on clock gene–driven oscillators [64, 230]. The pineal gland of Zebrafish is just one of many tissues that endow a circadian clock [231]. Notably, there is increasing evidence that the Zebrafish *Aanat* gene is endogenously clock-controlled in the pineal gland [232–234].

In birds, clock genes have been found in many tissues [235–238], with endogenous oscillators seemingly restricted

Table 2. Circadian dynamics in the human pineal gland

Pineal parameter	Pattern	Comments	Literature
Aanat mRNA	–		52, 87
AANAT protein	–		87
p31T-AANAT protein	–		151
AANAT activity	∿		87
Asmt mRNA	–		52, 87
ASMT protein	–		151
ASMT activity	–		87
Melatonin content	∿	Similar in plasma, saliva, urine (metabolite)	39, 87, 93,166
Per1 mRNA	∿/–	Controversial observations	150, 182
PER1 protein	–	Nucleo-cytoplasmic shuttling	150
Cry1 mRNA	∿/–	Controversial observations	150, 182
CRY1 protein	–	Nucleo-cytoplasmic shuttling	150
Clock mRNA	–		150, 182
CLOCK protein	–	Nucleo-cytoplasmic shuttling	150
Bmal1 mRNA	∿/–	Controversial observations	150, 182
BMAL1 protein	–		150
β-adrenergic receptor mRNA	∿		182

AANAT, arylalkylamine *N*-acetyltransferase; ASMT, acetylserotonin *O*-methyltransferase. Listed are the so far analysed parameters in the human pineal gland that are directly or indirectly related to its chronobiological function. All presented results are derived from analyses using autoptic human pineal glands. For abbreviations, see text.

to the eyes, the SCN and the pineal gland. Avian pinealocytes are unique, as not only is the circadian oscillator preserved, but its cells are also photosensitive and thus can be directly entrained by light [239, 240]. Indeed, binding of clock gene heterodimers to the *Aanat* promoter is critical for the expression of AANAT at least in chicken [241, 242]. There is strong evidence that in nonmammalian vertebrates, clock gene products are involved in the modulation of rhythmic melatonin synthesis through binding to inherent E-Box elements in the *Aanat* gene promoter [115].

The mammalian pineal gland

Clock gene expression has been detected in various mammalian tissues. It seems that they have different functions in non-SCN tissues and organs, not necessarily linked to time-measuring capacities, but also as regulatory gene transcription factors [8, 243]. It is therefore of interest to understand which role clock genes and their protein products play in the pineal gland as a mammalian brain structure that has inherited the capacity to generate a time-of-day-dependent humoral signal, the circadian synthesis of melatonin. So far, only few mammalian species have been investigated with respect to clock gene expression in the pineal gland, and a functional link to melatonin synthesis is still lacking.

Rhythmic melatonin synthesis vanishes immediately after culturing a mammalian pineal gland, demonstrating the absence of a robust endogenous clock that is coupled to a distinct output. This experimentally based suggestion is confirmed, as even in pineal explants of *Per*-promoter::luciferase transgenic rats and mice, enzymatic reporter-gene activity rhythms dampened out rapidly and vanished after 3 days in culture [225, 226, 229]. Thus, individual mammalian pinealocytes seem to desynchronize upon deprivation from SCN input in contrast to many other tissues, in which clock gene promoter-driven reporter-gene rhythms continue to oscillate in culture for days and weeks [225, 226, 229].

Whether the presence of clock genes in the mammalian pineal gland is advantageous for the fine-tuning of melatonin synthesis, or whether they are phylogenetic remnants of the pineal gland's ancestral oscillator capacity, remains unclear [8]. While clock gene–deficient mice are generally helpful tools to understand their function, many of these knockout animals become arrhythmic, leading to an immediate cessation of rhythmic melatonin synthesis [57, 224]. Thus, only a conditional knockout of clock genes in the pineal gland can separate their systemic impact on circadian rhythm generation from their potential pineal-specific role. A possible causal relationship could be deciphered by comparing responses in the dynamics of pineal clock gene expression and in melatonin synthesis to a phase-shifting stimulus. The parallel temporal adaptation of the phases of clock gene expression and of melatonin levels would indicate a codependency of parameters, and a temporally dissociated re-entrainment would speak against a molecular interaction of these pineal elements.

Notably, there are major differences in clock gene expression and regulation in the pineal gland of rodents on the one hand and ungulates and primates on the other hand (Table 1), similar to what has been observed in the regulation of melatonin synthesis (Table 1; see section The mammalian pineal gland).

Rodents

Clock gene expression and levels of their protein products display clear rhythms in the rodent pineal gland, with dynamics only partly fitting into a self-regulating molecular clock [79]. The archetypical clock gene *Per1* is inducible by the activation of the cAMP-signalling pathway [228, 244, 245], likewise to its regulation in the SCN [224], and thus can potentially trigger the initiation of a clockwork cycle. Clock genes in the rodent pineal gland are rhythmically expressed under both conditions, a 12-hr light/12-hr dark lighting regimen and constant darkness. The circadian rhythm in the expression pattern is abolished by injection of an adrenergic antagonist such as propranolol [79, 80, 228, 244, 246], indicating that at least *Per1* is another cAMP-inducible pineal gene.

Notably, clock gene expression dynamics are independent of functional melatonin synthesis, as patterns in melatonin-deficient C57BL mice are very similar to those of melatonin-proficient C3H mice [79]. Clock gene expression in the rodent pineal gland seems to be age independent, as its nocturnal amplitude is not different between

young and old *Per1*-luciferase transgenic rats [247], which is in contrast to the documented decline in the amplitude of the nocturnal melatonin surge with age in rodents [248] and in man (see section: Melatonin synthesis in the mammalian pineal gland: Molecular details of human melatonin synthesis). In contrast to the observations made in lower vertebrates, the above-mentioned data are not in favour of a direct link between rhythmic melatonin synthesis and clock gene expression in the rodent pineal gland.

Sheep and monkey

In nonrodent mammalian species, clock genes and regulatory mechanisms were analysed in the pineal gland of the Soay sheep [106] and their presence documented in the foetal Capuchin monkey pineal gland [249]. In sheep, no rhythm in the expression of the clock gene *Cry1* was apparent [106], likewise to what had been seen for *Aanat* expression (see section: Melatonin synthesis in the vertabrate pineal gland: Sheep and monkey). Only when animals were kept under short days, i.e. when pinealocytes were exposed to a prolonged sympathetic stimulus during nighttime, a very low–amplitude rhythm for *Per1* was observed [106]. This principally constitutive clock gene expression in the sheep pineal gland supports the assumption that at least dynamics in their transcript abundance is not central for other pineal-specific rhythmic processes (Table 1). Notably, the mRNA for the transcription factor *Icer* is also constantly expressed in the sheep pineal gland over a 24-hr day/night period, contrasting the high-magnitude day/night differences in *Icer* expression observed in the rodent pineal gland [88, 95].

It thus may be a generalized phenomenon in the ungulate and the primate pineal gland that post-transcriptional mechanisms are much more important for rhythm generation when compared with signalling in this rodent brain structure. Whether constant mRNA levels in the pineal of these species are achieved by a tonic long-term transcriptional activator, or a high degree of mRNA stability over the 24-hr cycle, is not known.

Human

Human chronobiological dysfunctions often appear in conjunction with mutations in clock genes ([47, 223]; for details: see section Sleep phase syndromes), but notably, pineal melatonin synthesis is often affected as well ([93, 209]; see section: Disturbed melatonin synthesis, causes and curative countermeasures). However, the possible role of clock genes in the human pineal gland is difficult to dissect from general chronobiological dysfunctions for the above given reasons.

Using noninvasive or minimally invasive techniques, rhythms in human clock gene mRNAs have been demonstrated and assessed in skin, oral mucosa, serum and saliva [250–256]. Phase-shifting the human endogenous circadian clock by giving a light pulse to the eyes at night evokes a phase shift in the melatonin rhythm in saliva and also a phase shift in the rhythmic expression profile of the clock gene *Per2* in oral mucosa cells [256]. These experiments elegantly demonstrated the link between the master function of the central oscillator in the human SCN and peripheral 'slave oscillators'. It does not, however, provide a functional explanation for the role of clock gene expression in cells and tissues other than in the SCN. A significant link between human chronotype and transcriptional dynamics was recently shown in cultured human dermal fibroblasts [257]. This approach allows exact monitoring of the phase and amplitude of the endogenous SCN clockwork in different chronotypes by mirroring rhythms of clock gene expression in these peripheral cells. Simultaneously, another 'hand of the clock' can now be linked to the defined chronotype by recording dynamics in melatonin secretion of a given patient from serum or urine, eventually allowing accurate interventional treatments.

An analysis of clock gene expression in autoptic human pineal tissue provides a unique chance to, at least, temporaily (and possibly also causally) correlate the dynamics in human melatonin synthesis with dynamics of these novel time-measuring molecules. To address this lack of knowledge on rhythmic processes in the human brain, two recent studies have analysed the diurnal expression patterns of clock genes and their protein products in time-of-death-matched autoptic human pineal gland tissues [150, 182]. In both investigations, specimens were allocated into four almost identical time-of-deaths groups. As the main focus of the two studies was different, data are not directly comparable, with small diurnal differences in the expression of the clock genes *Bmal1*, *Cry1* and *Per1* found in one study [182], which were not reproduced in a second study [150]. In the latter study, the parallel analysis of clock gene mRNA and corresponding protein content with time-of-day in human pineal tissue revealed a total lack in dynamics of *Per1*, *Cry1*, *Bmal1* and *Clock* mRNA and of corresponding proteins, when values obtained in the four daytime groups (day, dusk, night, dawn) were compared with each other (Fig. 12), despite a rhythmic melatonin synthesis by this organ. Neither when Ackermann and colleagues extracted from their very large group of analysed specimen various age-matched subgroups, including a group with similar characteristics as the control group in the work by Wu et al. [182], or when selecting a subgroup of specimens with relative short PMIs, nor when deliberately specimens were selected with explicitly low daytime and high nighttime melatonin values, evidence for the existence of a rhythmic transcription of clock genes or for dynamics in protein content was detected [87]. These divergent observations are in notable contrast to the matching results with respect to melatonin synthesis (Table 2; [87, 179, 198]). An absent rhythm in clock gene transcription in the human pineal gland matches to the above-mentioned observations of an absent rhythm in *Aanat* and *Asmt* mRNAs in sheep [106, 258] and in primates (Table 1)[52, 87, 109]. It is of interest that a similar lack of rhythmic clock genes expression was found in human pituitary glands, despite an evident diurnal rhythm in adrenocorticotrophic hormone content in the same tissue (K.A., E.M., J.H.S., unpublished observations).

Immunohistochemical analyses of the subcellular distribution of clock genes revealed a time-of-day-dependent translocation of PER1, CRY1, CLOCK, and to a lesser extent also BMAL1, in the human pineal [150]. Notably,

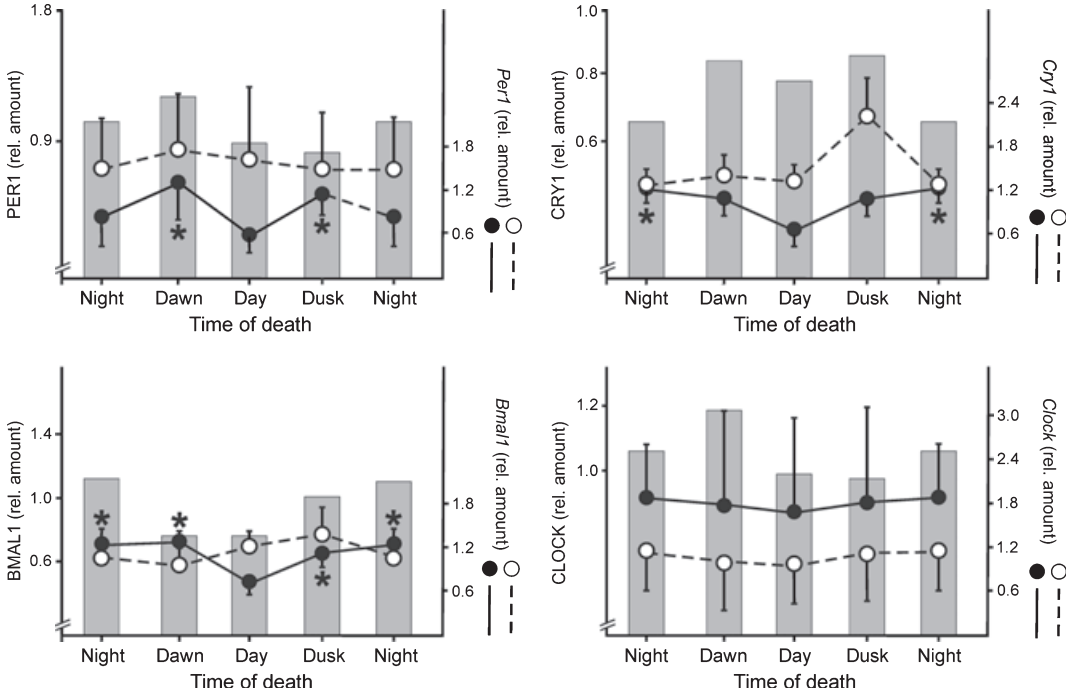

Fig. 12. Diurnal expression of clock genes in the human pineal gland. Results from two different studies on clock gene expression are compared ([150]: –○–; [182]: —●—). * Indicates significant differences between values. In addition, clock gene protein levels, as analysed in [150] are shown as grey columns (modified with permission from [150, 182]).

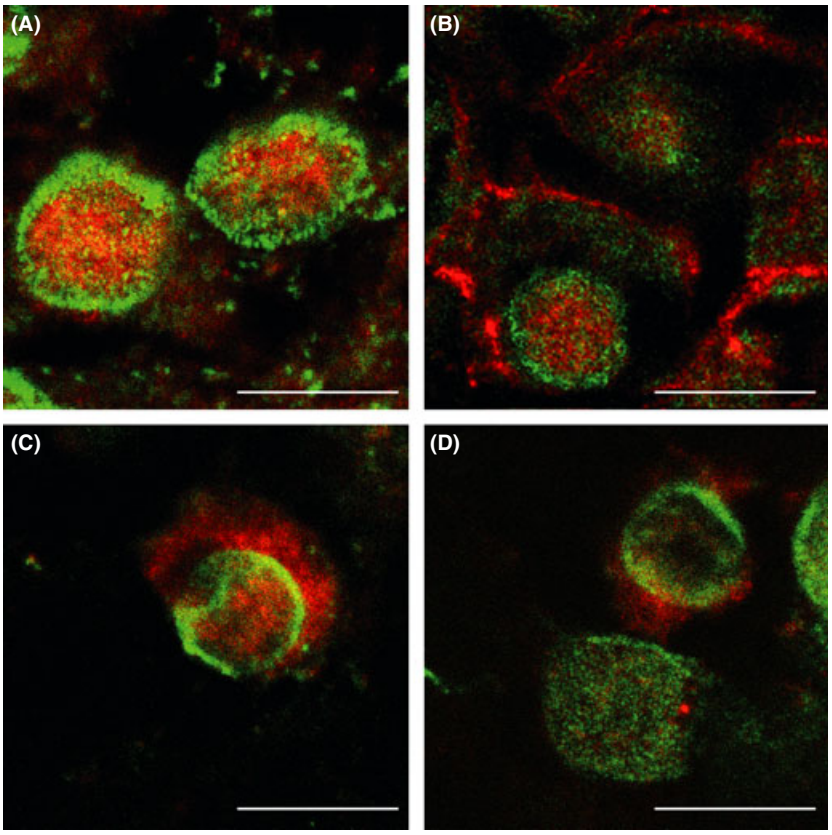

Fig. 13. Clock gene proteins in the human pineal gland. Immunoreaction of PER1 (A), CLOCK (B), CRY1 (C) and BMAL1 (D) (all in *red*) and of nuclear pore complex protein (*green*), visualized in 5-μm cryostat-cut sections of autoptic human pineal tissue, using confocal laser scanning microscopy. Scale bar: 10 μm (modified with permission from [150]).

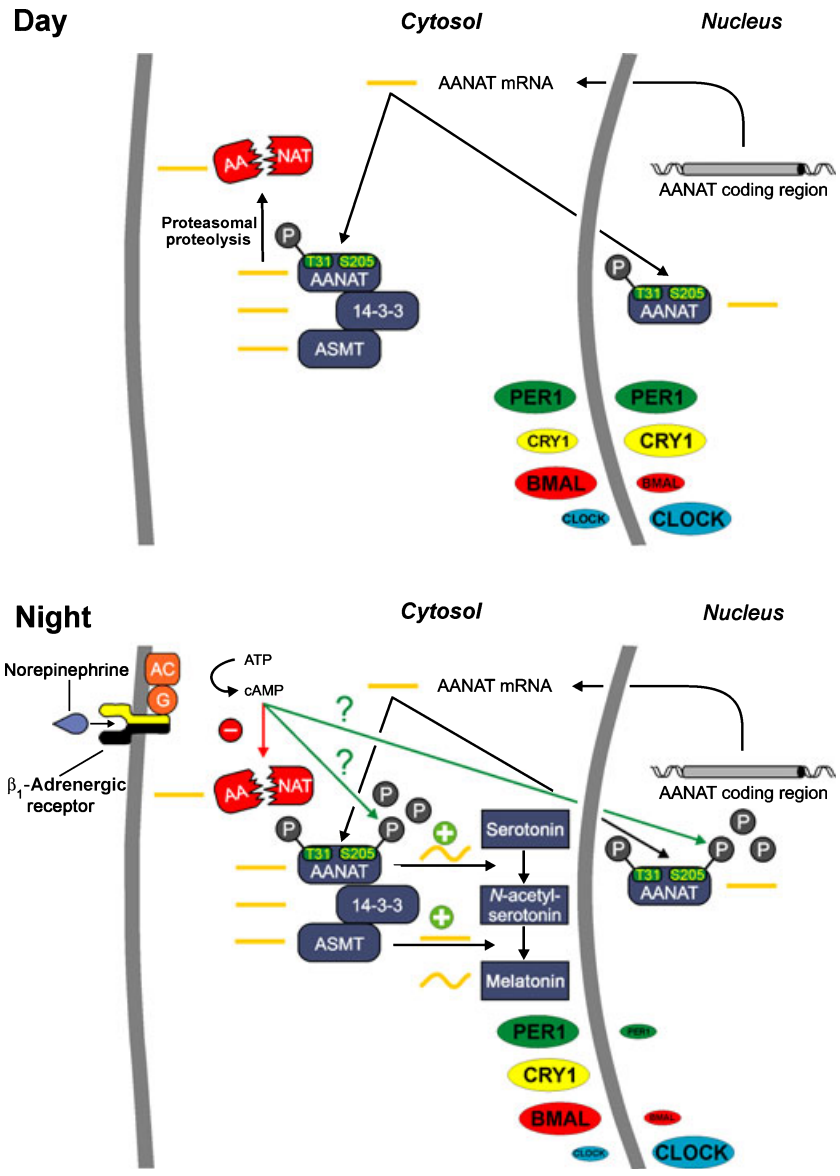

Fig. 14. Signalling events in the human pineal gland. Signal transduction pathways related to rhythmic melatonin synthesis in the human pineal gland, are depicted separately for day- and nighttime. Depicted pathways have evolved from integration of novel molecular details, as revealed by analysing autoptic human pineal tissue, with known neurophysiological and biochemical details. The schematic drawing suggests that phosphorylation of the arylalkylamine *N*-acetyltransferase (AANAT) at threonine residue 31 (p31T-AANAT) within a constitutively present p31T-AANAT/14-3-3/acetylserotonin *O*-methyltransferase complex determines p31T-AANAT activity and thus the nocturnal increase in melatonin synthesis in man. Note that according to this working hypothesis, the proteasomal proteolysis is not the only rate-limiting step for the rhythmic hormone production in man. The daytime-dependent amount of clock gene proteins in the cytosol or in the nucleus, respectively, is indicated by the size of the ellipses, with their function not known as yet. For details, see text. Abbreviations: AC, adenylyl cyclase; G, G-protein; P, phosphate group; S205, serine residue 205; T31, threonine residue 31.

such a 'nucleo-cytoplasmic shuttling' [258] appeared simultaneously for PER1 and CRY1 as expected, as heterodimerization of these two clock gene proteins is a prerequisite for imposing their negative feedback onto the clockwork [224, 259]. CLOCK protein was localized in the nucleus of pinealocytes at all times, which is consistent with the finding that CLOCK binds constitutively to E-box elements within a nuclear transcriptional complex in a BMAL1-dependent fashion [227, 260]. The nighttime localization of CLOCK at the cell membrane (Fig. 13) in human pinealocytes awaits further analysis to elucidate its functional significance (Fig. 14). The observed phenomenon of nucleo-cytoplasmic shuttling in the human pineal gland has been described earlier for other clock gene proteins in mouse fibroblasts [258, 260–263] and was shown to be driven by nuclear import and export signals that allow active transport from the nucleus to the cytoplasm and vice versa, without the need of *de novo* protein synthesis [258].

The recent data on clock gene expression in the human pineal gland showed that the well-known evolutionary conversion of this organ from the light-sensitive circadian pacemaker in lower vertebrates into an SCN-dependent secondary oscillator in mammals has also impaired rhythmic gene expression in this structure (Fig. 14, Tables 1 and 2). Cycling transcription of defined clock genes may play an important role in the rodent pineal gland but seems to have an ever fading relevance in the pineal gland of ungulates, primates, including man (Table 1). As indicated earlier, this evolutionary divergence in the importance of a transcriptional control of pineal function may be linked to the different lifestyles of rodents when compared with ungulates and primates.

Concluding remarks

While the human pineal gland was considered for long as an insignificant rudiment of phylogeny, our scientific knowledge had to be revised on the basis of elementary anatomical investigations [10, 264]. In the meantime, we have learned that in the human pineal gland, the neuro-

hormone melatonin serves the need to communicate time-of-day from the mother to the foetus and that manipulation of the hormone synthesis can efficiently prevent or even cure chronobiological misalignment of internal rhythms, as evoked by shift work, owing to transmeridian flights and in blind people. Moreover, the human melatonin profile and the activity and/or structure of elements of the hormone's synthesis pathway are often altered in diseases linked to the circadian system. Particularly, the recent detection of polymorphisms of clock genes as the cause for major sleep disorders makes the well-known phase-shifting capacity of melatonin an efficient drug of choice. Moreover, melatonin has not yet shown any side effects when taken orally as a chronobiotic agent. On the contrary, we know melatonin for its effectiveness to ease discomfort associated with blindness and jet lag, whether society inflicted or because of travel [139, 140].

Using melatonin as a pharmacotherapeutic or even as a preventative for chronobiological disturbances in the human requires a full understanding of the signalling routes responsible for the generation of the rhythmic hormonal signal in the pineal gland (Fig. 14) While it is tempting to transpose findings regarding molecular details within pineal signalling from animal models to human biology, the here presented findings demonstrate vividly the caveats to be considered. The 'enigmatic' structure of the human *glandula pinealis* still bears open questions and will remain an excellent model system for the study of signalling routes in neuroendocrine brain tissue.

Acknowledgements

The authors thank R. Bux, M. Enders, G. Kauert and H. Bratzke of the Institute of Forensic Medicine for support with material acquisition, A. Langhagen, N. Molotkov and A. Rami for excellent technical assistance, B. v. Schemm for help with graphics and M.Y. Storck and S. Schotten for continuous support. This work was supported by Arthur-und-Margarete-Ebert-Stiftung, August Scheidel-Stiftung, Heinrich und Fritz Riese-Stiftung, Hertie-Stiftung, and Stiftung Forensisches Forum.

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