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Generation of the Melatonin Endocrine Message in Mammals: A Review of the Complex Regulation of Melatonin Synthesis by Norepinephrine, Peptides, and Other Pineal Transmitters

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***Abstract*——Melatonin, the major hormone produced by the pineal gland, displays characteristic daily and seasonal patterns of secretion. These robust and predictable rhythms in circulating melatonin are strong synchronizers for the expression of numerous physiological processes in photoperiodic species. In mammals, the nighttime production of melatonin is mainly driven by the circadian clock, situated in the suprachiasmatic nucleus of the hypothalamus, which controls the release of norepineph-**

rine from the dense pineal sympathetic afferents. The pivotal role of norepinephrine in the nocturnal stimulation of melatonin synthesis has been extensively dissected at the cellular and molecular levels. Besides the noradrenergic input, the presence of numerous other transmitters originating from various sources has been reported in the pineal gland. Many of these are neuropeptides and appear to contribute to the regulation of melatonin synthesis by modulating the effects of norepinephrine on pineal biochemistry. The aim of this review is firstly to update our knowledge of the cellular and molecular events underlying the noradrenergic control of melatonin synthesis; and secondly to gather together early and recent data on the effects of the nonadrenergic transmitters on modulation of melatonin synthesis. This information reveals the variety of inputs that can be integrated by the pineal gland; what elements are crucial to deliver the very precise timing information to the organism. This also clarifies the role of these various inputs in the seasonal variation of melatonin synthesis and their subsequent physiological function.

I. Introduction

The pineal gland (or epiphysis) was probably described for the first time by Herophile, in the third century. He attributed to it the role of a sphincter regulating the flow of thought in the ventricular system of the brain. Some 450 years later, Galen observed that the pineal structure appeared different to that of nervous tissue but very similar to that of the other glands. It was described more precisely during the Renaissance through the documents of da Carpi, Vesalius, and Vesal. During this period, the prevailing concept was that ventricles contained the animal spirits. Nevertheless, these authors admitted that the pineal gland could not control these flows between ventricles III and IV. Vesal later considered the gland as the center of a fine vascular system, which in turn must have influenced Descartes.

The pineal gland was studied intensively by Descartes during the 17th century. He described the pineal gland as the third eye, not by analogy to its role in the control of the photoperiod, which he had no knowledge of, but because it is, in the Cartesian dualist vision, the place in the body where the soul exerts its control (the seat of imagination and common sense), and not the seat of the soul as it has often been referred to. "The reasonable soul," according to Descartes, "is lodged in the body, but not only as a pilot on its ship, it is necessary that it is united with its body." Descartes was the first to propose a "physiological" explanation for the functioning of the central nervous system, including the pineal gland, for the perception of the environment. Even if this Cartesian model appears a posteriori an unreliable model, this concept nevertheless prevailed for the next 250 years.

At the end of the 19th century Ahlborn and Rabl-Ruckhardt, then Graaf, Korschelt, and Spencer, described the anatomy, histology, innervation, and embryology of the mammalian pineal gland and noticed its resemblance to the epiphysis organ of lower vertebrates. In 1905, Studnicka established that phylogenetically the pineal gland derived from a photoreceptor organ, but its function remained unknown.

At the beginning of the 20th century the physiological role of the pineal gland was studied. Heubner presented the case of three girls with pineal tumors and precocious puberty. He concluded that the destruction of the pineal by the tumor had prevented the normal production of an antigonadotropic pineal hormone and raised the hypothesis that the pineal may control the onset of puberty. The link between the pineal gland and reproduction was thus established. In 1943, Bargman suggested that the endocrine function of the pineal gland was regulated by light, via the central nervous system.

From the 1970s, the number of publications on the pineal gland markedly increased. The first international congress that brought "pinealogists" together was held in 1965 in Amsterdam. Research on the pineal gland developed in four main directions.

1. Structure and ultrastructure: The pineal gland was described in numerous vertebrate species. In most mammals, it forms a solid mass located between the habenular and posterior commissures, but in rodents the pineal gland migrates dorso-caudally during ontogenesis, leading to a characteristic three-part gland (deep, stalk, and superficial gland; see Fig. 1 in the rat). Electron microscopy has allowed the fine description of pineal cells and their different phenotypes, as well as the ontogenesis and phylogenesis of the gland.
2. Innervation of the gland: The first description of nervous fibers in the pineal gland was made by Studnicka in the beginning of this century. The sympathetic innervation was described by Cajal in 1911 in the mouse. Since then, a complex innervation of the mammalian pineal gland has been described arising from various central and peripheral neural structures.
3. Histochemistry and biochemistry of the gland: Since the work of McCord and Allen, in 1917, it was assumed that a substance contained in the pineal gland was responsible for the bleaching of amphibian skin. In 1958, Lerner et al. identified this substance as *N*-acetyl-5-methoxytryptamine and named it melato-

nin (MEL[1]) by analogy to its effect on amphibian skin. The different enzymes involved in MEL synthesis were then identified. Their regulation by various pineal transmitters is still under investigation. Other indolic and nonindolic substances have also been identified in the pineal gland.

4. Endocrine function of the gland: In 1954, Kitay and Altschule demonstrated that the pineal gland influences reproductive function. Discovery of the link between the light/dark (L/D) cycle and the metabolism of the pineal gland was a milestone in the history of understanding the endocrine function of the pineal gland. Today, the target tissues and the mechanisms of action of MEL on the reproductive axis are still not totally understood. In addition, recent investigations have revealed that MEL displays widespread effects in the organism, for example on the hypothalamic circadian clock, the immune system, or in the retina. In addition, MEL's antioxidant properties and its ability to modulate neurotransmission show less specific and ubiquitous effects.

[1]Abbreviations: MEL, melatonin; L/D, light/dark; SCN, suprachiasmatic nucleus of the hypothalamus; VP, vasopressin; IR, immunoreactive; IGL, thalamic intergeniculate leaflet; PT, pars tuberalis of the adenohypophysis; MEL-R, melatonin receptor; SP, short photoperiod; LP, long photoperiod; DA, dopamine; 5-HT, 5-hydroxytryptamine (serotonin); NE, norepinephrine; ACh, acetylcholine; NK, neurokinin/tachykinin family; MT_1, melatonin receptor of subtype 1; MT_2, melatonin receptor of subtype 2; AC, adenylate cyclase; DAG, diacylglycerol; IP_3, inositol triphosphate; PKC, protein kinase C; CRE, cAMP response element; CREB, CRE-binding protein; RHT, retino-hypothalamic tract; PACAP, pituitary adenylate cyclase activating peptide; sP, substance P; NPY, neuropeptide Y; Enk, enkephalin; VIP, vasoactive intestinal peptide; GRP, gastrin-releasing peptide; SOM, somatostatin; PVN, hypothalamic paraventricular nucleus; OT, oxytocin; IML, intermediolateral column of the spinal cord; SCG, superior cervical ganglion; PHI, histidine isoleucine peptide; CGRP, calcitonin gene-related peptide; TH, tyrosine hydroxylase; SCGx, superior cervical ganglionectomy; HRP, horseradish peroxidase; HCRT, hypocretin; LHRH, luteinizing hormone-releasing hormone; DSIP, delta-sleep inducing peptide; ISH, in situ hybridization; RT-PCR, reverse transcription-polymerase chain reaction; CNP, C-type natriuretic peptide; SN, secretoneurin; αMSH, melanin-stimulating hormone of type α; 5-HTP, 5-hydroxytryptophan; TPOH, tryptophan hydroxylase (EC 1.14.16.4); AAAD, aromatic amino acid decarboxylase (EC 4.1.1.28); HIOMT, hydroxyindole-*O*-methyltransferase (EC 2.1.1.4); MAO, monoamine oxidase (EC 1.4.3.4); 5-HIAL, 5-hydroxyindole acetaldehyde; 5-HIAA, 5-hydroxyindole acetic acid; 5-MIAA, 5-methoxyindole acetic acid; 5-HL, 5-hydroxytryptophol; 5-ML, 5-methoxytryptophol; AA-NAT, arylalkylamine-*N*-acetyltransferase (EC 2.3.1.37); NAS, *N*-acetylserotonin; GC, guanylate cyclase; PKA, cAMP-dependent protein kinase; CaM, calmodulin; $PKCa^{2+}$/CaM, Ca^{2+}/calmodulin-dependent protein kinase; *p*-CPA, *para*-chlorophenylalanine; NAT, arylamine-*N*-acetyltransferase (EC 2.3.1.5.); CATBP, CCAAT box-specific binding proteins; P-CREB, phosphorylated form of CREB; nat-CRE, CRE-like sequence specific of the *Aa-nat* gene promoter; PIRE, pineal regulatory element; CRX, cone-rod homeobox; AP-1, activating protein 1; D/D, constant dark; L/L, constant light; β_1-AR, adrenergic receptor of subtype β_1; ISO, isoproterenol; PROP, propranolol; α_1-AR, adrenergic receptor of subtype α_1; α_2-AR, adrenergic receptor of subtype α_2; PLC, phospholipase C; NO, nitric oxide; NOS, NO synthase; $Ca^{2+}{}_i$, intracellular calcium; MAPK, mitogen-activated protein kinase; IEG, immediate early gene; CREM, CRE modulator; ICER, inducible cAMP early repressor; AR, adrenergic receptor; $VPAC_2$-R, type 2 VIP/PACAP receptor; $VPAC_1$-R, type 1 VIP/PACAP receptor; PAC_1-R, PACAP specific receptor; PP, pancreatic peptide; Y_n-R, NPY receptor of subtype *n* (n = 1–5); Y_6-R, NPY receptor of subtype 6. OT-R, oxytocin receptor; SST1, group 1 SOM receptors (sst2, sst3, sst5); SST2, group 2 SOM receptors (sst1 and sst5); NKA, neurokinin A; NKB, neurokinin B; CT, calcitonin; HCRT-1, 33-amino acid form of hypocretin; HCRT-2, 28-amino acid form of hypocretin; ANP, A-type natriuretic peptide; BNP, B-type natriuretic peptide; CNP, C-type natriuretic peptide; GC-A, ANP and BNP receptor; GC-B, CNP receptor; Ang II, angiotensin II; mACh-R, muscarinic cholinergic receptor; nACh-R, nicotinic cholinergic receptor; MV, microvesicle; CT, circadian time; ZT, Zeitgeber time; BIBP3226, (*R*)-N_2-(diphenylacetyl)-*N*-[(4-hydroxyphenyl)methyl]-D-argininamide.

The objective of this review is to consolidate and update our current knowledge of the complex and varied inputs controlling the rhythmic synthesis of MEL in the mammalian pineal gland.

II. Role of Melatonin

MEL is secreted by the pineal gland with daily and seasonal rhythms mainly under the control of the circadian oscillator located in the suprachiasmatic nuclei of the hypothalamus (SCN). This hormone, which is released at night with duration inversely proportional to the duration of the photoperiod, participates in the transmission of the circadian and seasonal message to the organism (see Reiter, 1993; Goldman, 1999 for reviews). For many years, but especially during the last decade, many studies have been performed to understand the physiological role, sites, and mechanisms of action of MEL.

A. Regulation of Seasonal Rhythms

The pineal gland is a major component of the endocrine system that allows mammals to respond to the annual changes in photoperiod by adaptive alterations of their physiological state. The best example of such photoperiod-dependent physiological functions is the activation/inactivation of the reproductive axis, a phenomenon in which the pineal and its MEL rhythm are essential. Numerous studies have now demonstrated that the pineal gland is a neuroendocrine transducer receiving photoperiodic information from the retina and circadian SCN oscillator, and transmitting this to the reproductive system via a particular dynamic pattern of MEL secretion (see Hoffmann, 1979; Reiter, 1980; Goldman and Darrow, 1983; Bittman, 1984; Tamarkin et al., 1985; Pévet, 1988; Goldman, 2001 for reviews). However, several fundamental questions remain before the role of MEL in the regulation of seasonal function is elucidated: 1) where is the photoperiodic information encoded before its translation into the MEL rhythm? 2) Where and how is the MEL rhythm decoded to regulate specific seasonal functions? 3) Which parameters of the MEL rhythm (phase, duration, amplitude, or total quan-

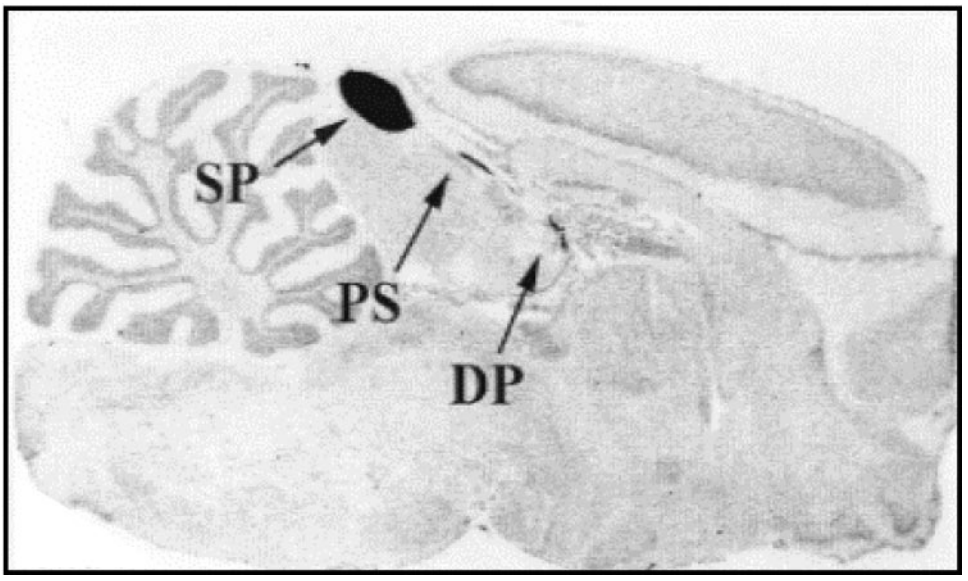

FIG. 1. Autoradiogram of a parasagittal section of rat brain hybridized with *Hiomt* antisense cRNA. *Hiomt* mRNA is expressed in the three parts of the pineal complex: SP, superficial pineal; PS, pineal stalk; DP, deep pineal; original magnification, 6× (from Ribelayga et al., 1998, with permission).

tity) are interpreted as the photoperiodic message by the target structures?

Recently, data have accumulated that strongly suggest that the hypothalamic circadian clock may be the site for the integration of annual changes in photoperiod (see Goldman, 2001; Schwartz et al., 2001 for reviews): namely, a circadian reading of the photoperiod appears necessary (Maywood et al., 1990); FOS reactivity in the SCN following a light stimulus depends on the photoperiod history (Sumova et al., 1995; Vuillez et al., 1996); clock gene expression in the SCN displays MEL-independent photoperiodic variations (Messager et al., 1999b, 2000, 2001; Nuesslein-Hildesheim et al., 2000); and the daily profile of vasopressin (VP) mRNA differs in long and short photoperiods (Jac et al., 2000). In addition, the thalamic intergeniculate leaflet (IGL), a relay between the retina and SCN, may be involved in photoperiod integration (Menet et al., 2001).

Several neural structures have been identified as targets for MEL's effect on seasonal function. The pars tuberalis of the adenohypophysis (PT), containing the highest density in MEL receptors (MEL-R), is the site of action for MEL regulation of prolactin secretion (see Lincoln, 1994; Malpaux et al., 1995, 2001; Hazlerigg et al., 2001 for reviews) and displays MEL-dependent daily and photoperiodic variations in clock gene expression with lower amplitude under a short photoperiod (SP) (Messager et al., 1999b, 2000, 2001; von Gall et al., 2002a). Identification of the specific molecule released from the PT in response to MEL, which acts on the lactotrophs, named tuberalin, remains unknown, although two 21- and 72-kDa proteins were recently identified in the bovine PT (Guerra and Rodriguez, 2001). Depending on the species, various hypothalamic sites (SCN in Siberian hamster; mediobasal hypothalamus in Syrian hamster, premammillary hypothalamus in sheep) are MEL targets for the specific control of reproductive function (Badura and Goldman, 1992; Maywood and Hastings, 1995; Malpaux et al., 1998). Although it has been clearly shown that MEL is the photoperiodic endocrine message for each structure, it has not yet been elucidated how this MEL message is decoded at the cellular level. Several studies have reported that, although MEL is an acute inhibitor of cAMP accumulation, tissues pre-exposed to long-duration (up to 16 h) MEL treatment become hypersensitive to cAMP (Hazlerigg et al., 1993; Witt-Enderby et al., 1998; Messager et al., 1999a; Pelisek and Vanecek, 2000) or cAMP elevating agents like adenosine (von Gall et al., 2002a) even with a lower number of MEL-R.

To define which parameters of the MEL secretion pattern (phase, duration, amplitude, or total quantity) are interpreted as a photoperiodic message by the target structures, several hypotheses have been proposed from analysis of the endogenous MEL patterns in different conditions and from studies with acute injections or chronic infusions of exogenous MEL (Fig. 2). Observations of the MEL secretion pattern in various species raised in different photoperiodic conditions have shown that the duration of the nocturnal MEL peak is positively related to the length of the night (sheep: Rollag and Niswender, 1976; Karsch et al., 1988; rat: Illnerova and Vanecek, 1980; Siberian hamster: Illnerova et al., 1984; Ribelayga et al., 2000; Syrian hamster: Skene et al., 1987; Maywood et al., 1993; Miguez et al., 1995a; European hamster: Vivien-Roels et al., 1992). Furthermore, experiments using acute injections or constant infusion of MEL have shown that the duration of a high circulating MEL level is the limiting factor to obtain a photoperiodic response (see Carter and Goldman, 1983;

TABLE 1
Characteristics of the various peptides present in the rodent pineal gland

Peptide	a.a.	Origin			Content	Effect on melatonin synthesis pathway								Receptor	EC_{50}	Variations	
		Neural	Endocrine	Intrapineal		cAMP	cGMP	Ca^{2+}	TPOH	AA-NAT	HIOMT	MEL	5-HT	NE		Daily	Seasonal
VIP	28	+			17 pmol/g	+	+	0/+	+	+	+	+	+		$VPAC_1$	0.1 nM	Yes	
PACAP	27/38	+			20 pmol/g	+	0	0/+	+	+	+	+	+		$VPAC_1$; PAC_1	0.1 nM	Yes/no	
NPY	36	+		+[a,b]	430–785 pmol/g	–					+	0/+/−/x	+	–	Y_1(post); Y_2(pre)	5/50 nM	Yes	Yes[g]
VP	9	+	+	+[c]	20 fmol/gland	x		0/+		x		x			V_{1a}	7 nM	Yes	Yes
OT	9	+	+	+[d]	14 fmol/gland	x						x					Yes	Yes
SOM	14/28	+		+	1–3 ng/mg prot	0				0		0			SST2		Yes	Yes
SP	11	+				0				0		0			NK_1			
CGRP	37	+																
SN	33	+		+[e]	34 fmol/gland			–				–	–				No	
HCRT1/2	28/33									–		–			Orexin-2			
DSIP	9		+			0			+			+	+					
CNP			+	+[f]			+					0						
Opioids		+		+														
α-MSH	13		+	+	180 pg/gland	–											Yes	
LHRH		+	+								+	+						Yes

Effects: +, stimulating; –, inhibiting; 0, no effect; x, potentiating effect.
[a] Bat.
[b] Syrian hamster.
[c] ARNm but no peptide.
[d] Cow.
[e] Syrian hamster.
[f] Cow.
[g] European hamster.
Nonspecified: rat.

Pitrosky et al., 1991; Bartness et al., 1993 for reviews). Consequently, the *duration* of the nocturnal MEL peak is an important factor for the transmission of photoperiodic information from the environment to the body. The early experiments showed that an acute injection of MEL at the end of the day or beginning of the night to intact hamsters kept in long photoperiod (LP) induced gonadal regression, while a similar injection made at the end of the night or at the beginning of the day had no effect. This observation led to the hypothesis that the *coincidence* of the injection of MEL with a phase of sensitivity was a deciding factor for the appearance of a physiological effect (see Tamarkin et al., 1976; Reiter, 1987 for review). Recently, a study performed in our laboratory (Pitrosky et al., 1995) has shown that the photoperiodic response to MEL in the Syrian hamster depends on a phenomenon of coincidence. The infusion of two consecutive MEL peaks, whose length from the beginning of the first peak to the end of the second peak corresponded to an SP signal but whose total quantity of infused MEL corresponded to an LP signal, induced an SP-type response of the reproductive axis. The physiological response thus depends on the interval between the first and the second MEL peak but not at the clock time when the double MEL peak is applied.

In addition, the *amplitude* of the nocturnal peak of MEL could also be an important parameter in photoperiodic transmission (see Vivien-Roels, 1999 for review). Several examples of photoperiodic variation in the amplitude of the MEL peak have been observed, for example, in the pig (McConnell and Ellendorf, 1987; Taste et al., 2001), mule (Cozzi et al., 1991), Siberian hamster (Lerchl and Schlatt, 1992; Steinlechner et al., 1995; Miguez et al., 1996; Ribelayga et al., 2000), European hamster (Vivien-Roels et al., 1992, 1997), and horse (Guérin et al., 1995). Annual variations in the amplitude of the nocturnal MEL peak are especially visible when animals are maintained in their natural environment. These observations suggest that factors other than the photoperiod that display annual variations (e.g., temperature, quality/quantity of food, humidity) may be integrated by the organism and transmitted via the secretion of MEL (Pévet, 1987; Pévet et al., 1991; Vivien-Roels, 1999). These other nonphotic environmental factors could modulate the perception of the photoperiod by altering the metabolism of the pineal gland. Environmental temperature seems an important factor since diminution of the temperature accelerates gonadal regression in Siberian and Syrian hamsters placed in SP (Heldmaier and Steinlechner, 1981; Pévet et al., 1986; Larkin et al., 2002). In addition, a decrease in temperature 1) increases enzyme activity in the rat pineal gland (Nir et al., 1975); 2) increases the amplitude of the nocturnal pineal MEL peak in the Syrian hamster (Brainard et al., 1982, but discussed by Pévet et al., 1989a) and European hamster (Vivien-Roels et al., 1997); and 3)

modulates the inhibitory effect of light applied at night (Stieglitz et al., 1991). Currently, anatomical structures and transmitters involved in these effects of temperature are not known and could act directly on the pineal gland or on intermediate structures sensitive to the temperature.

Historically considered as a pro or antigonadotropic hormone, according to species, it is clearly established now that MEL is a pivotal endocrine messenger used to time several annual functions with the seasonal cycle to ensure adaptation and survival of individual in their cyclic environment.

B. Regulation of Circadian Rhythms

In all mammals studied to date, whether they exhibit nocturnal or diurnal activity, MEL is synthesized in the pineal gland during the dark phase of the light/dark cycle and is rapidly delivered to the body via the bloodstream. Pinealectomy does not alter the animal's circadian rhythm in rest-activity but facilitates the re-synchronization of the animal to a new photoperiod (Cheung and McCormack, 1982). The daily rhythm of MEL is considered to be a circadian mediator used by the endogenous SCN clock to deliver the circadian message to MEL target structures (containing MEL-R). In addition, MEL exerts a "chronobiotic" effect by acting directly on the SCN, which contain MEL-R (Vanecek et al., 1987), to affect the circadian clock (see Pévet et al., 2002 for review).

In rats and hamsters with free-running circadian rhythms, pharmacological doses of exogenous MEL are capable of synchronizing the circadian rhythms of locomotor activity and MEL synthesis (see Redman et al., 1983; Armstrong and Chessworth, 1987; Humlova and Illnerova, 1990; Kirsch et al., 1993; Drijfhout et al., 1996b; Grosse and Davis, 1998; Pitrosky et al., 1999;

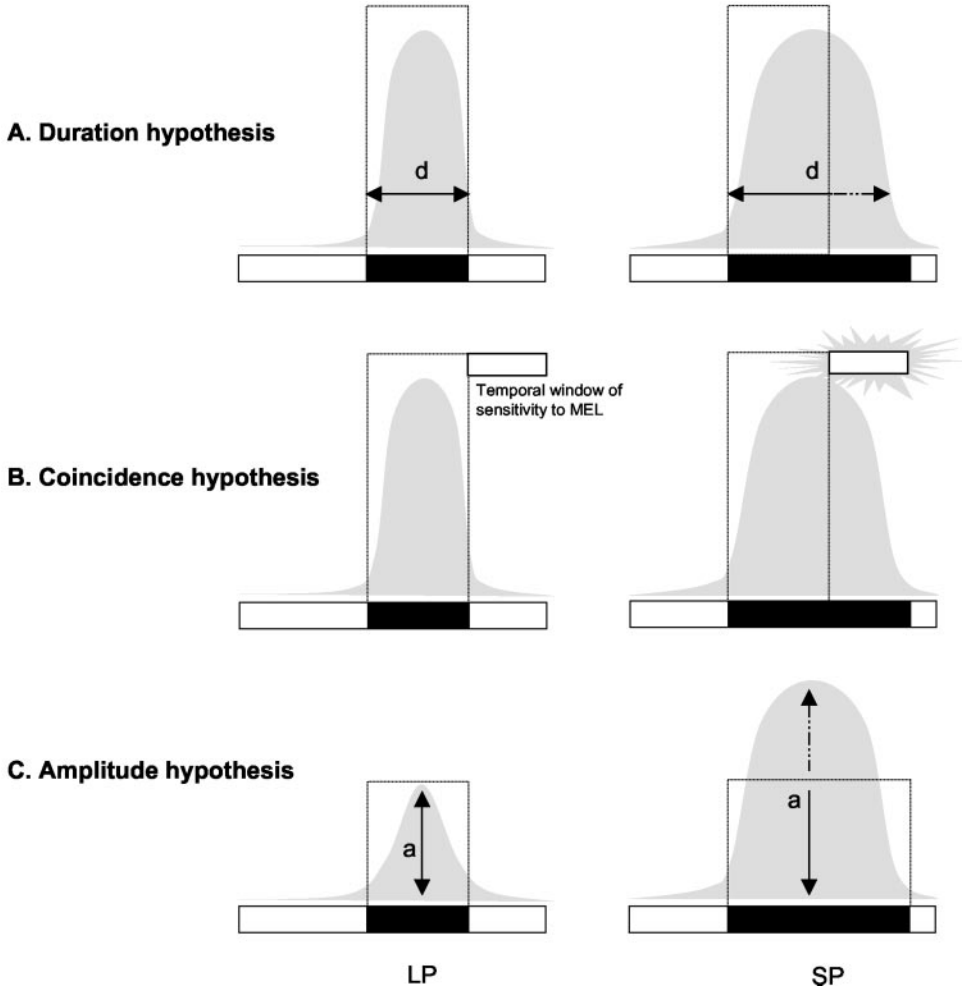

FIG. 2. Schematic representation of the different theoretical models explaining how the photoperiodic MEL endocrine message is decoded. In response to a change in the photoperiod, the daily MEL profile displays substantial changes, primarily affecting the duration and/or the amplitude of the nocturnal peak. Distortion of the MEL message, in turn, has an impact on many physiological functions. How the organism "reads" the modifications of the MEL profile is still largely hypothetical and appears species-dependent. The duration of the nocturnal peak seems to be an important parameter in many photoperiodic species. Photoperiodic dependent changes may rely on the absolute duration of the nocturnal MEL peak (A) or on the presence of a time-window of sensitivity to MEL (B). In addition, in some species, the amplitude of the nocturnal MEL peak may be an important parameter (C). a, amplitude of the nocturnal MEL peak; d, duration of the nocturnal peak of MEL; LP, long photoperiod; SP, short photoperiod.

Schuhler et al., 2002; Pévet et al., 2002 for review). The synchronizing effect of MEL occurs at a particular circadian time, being different according to species (e.g., beginning of the active period, CT 12, in the rat). Recently, it was reported that exogenous MEL, applied directly into the SCN by reverse microdialysis, not only phase-advances the endogenous MEL peak but also increases the amplitude of the MEL peak (Bothorel et al., 2002). Additionally, various in vitro studies have demonstrated a local effect of MEL on SCN metabolism, electrical activity, and circadian rhythmicity (Cassone et al., 1988; Stehle et al., 1989; Mc Arthur et al., 1991). At the moment, it is not known why high doses of exogenous MEL are necessary to induce a phase-shifting effect. MEL may exert its synchronizing properties indirectly on clock inputs or clock outputs, or directly on the clock via MEL-R (MEL-R were identified on VP-containing SCN neurons; Song et al., 1999) or other binding sites (see Pévet et al., 2002 for review). This property of MEL is used, along with several circadian signals, between the mother and fetus to entrain the circadian clock of the offspring (Reppert et al., 1979; Reppert and Weaver, 1991).

In humans, this "chronobiotic" property of MEL has been used to help re-synchronize individuals showing disrupted circadian rhythms, for example, related to "delayed sleep phase" syndrome, jet-lag, night shift work, or in some blind people (Arendt et al., 1984, 1987, 1988, 1997; Lewy et al., 1992; Claustrat et al., 1995; Skene et al., 1996; Lockley et al., 2000; Takahashi et al., 2000).

C. Other Roles of Melatonin

1. Autocrine/Paracrine Effects. In addition to the pineal gland, MEL is synthesized in several other structures (retina, Harderian gland, gut) where the genetic expression and biochemical activity of the MEL-synthesizing enzymes have been detected (Quay, 1965; Cardinali and Wurtman, 1972; Quay and Ma, 1976; Brammer et al., 1978; Pévet et al., 1980a; Vivien-Roels et al., 1981; Gauer and Craft, 1996; Roseboom et al., 1996; Ribelayga et al., 1998a; Djéridane et al., 1998, 2000). Since following pinealectomy the plasma MEL concentration is very low and since some of these structures contain MEL-R (Dubocovich and Takahashi, 1987; Lopez-Gonzalez et al., 1991), it has been proposed that MEL plays an auto/paracrine role in these structures.

In the *retina*, MEL is rhythmically synthesized in the photoreceptors in a circadian manner (see Cahill and Besharse, 1995 for review), which persists in vitro in constant conditions (Tosini and Menaker, 1996, 1998). MEL alters various aspects of retinal metabolism (see Iuvone, 1996 for review). Most of the retinal effects of MEL are indirect, and probably consist primarily in the inhibition of dopamine (DA) release from amacrine cells (Dubocovich, 1983). Conversely, DA acutely inhibits MEL synthesis in the retina and affects the phase of the MEL rhythm (Iuvone et al., 1987; Nguyen-Legros et al., 1996; Jaliffa et al., 2000; Tosini and Dirden, 2000).

The rodent *Harderian gland* also synthesizes MEL but the mechanisms regulating the synthesis and local effects of the hormone are still not well understood (Djéridane et al., 1998, 2000).

In the *pineal gland* several observations also suggest that MEL exhibits autocrine/paracrine effects. For example, in neonate but not adult rats, the pineal gland displays MEL-R binding (Zitouni et al., 1995). Exogenous MEL modifies various morphological and biochemical pineal parameters, namely proteic microtubules (Freire and Cardinali, 1975), enzymatic activities (Freire and Cardinali, 1975), presynaptic release of neurotransmitters (Chuluyan et al., 1991), and pre and postsynaptic release of the MEL precursor serotonin (5-HT; Miguez et al., 1995b).

2. Modulation of Neurotransmission It has been proposed that MEL could, on one hand, alter the release of several neurotransmitters, especially DA, 5-HT, norepinephrine (NE), acetylcholine (ACh) and, on the other hand, could modulate the postsynaptic response (Cardinali et al., 1975; Carneiro et al., 1994; Markus et al., 1996; Bucher et al., 1999). For example, MEL potentiates the NE-induced vasoconstriction of the rat caudal artery (Bucher et al., 1999). In addition, MEL, through activation of its different receptor subtypes, can differentially modulate the function of type A γ-aminobutyric acid ($GABA_A$) receptors (Wan et al., 1999). It has been proposed that some effects of exogenous MEL in humans (sedative, analgesic, anticonvulsive, anxiolytic) could be related to its interaction with the GABAergic system (Golombek et al., 1996).

3. Effects of Melatonin on the Immune System. Earlier studies reporting that pinealectomized rats displayed a structurally modified thymus and that MEL treatment or pineal grafting prevented thymic involution in very old mice led to the concept that MEL could affect the immune system (see Provinciali et al., 1996; Liebmann et al., 1997; Reiter et al., 2000a; Maestroni, 2001 for reviews). In vivo, high exogenous doses of MEL show a general stimulation of the immune system. It increases T cell activity, lymphocyte growth, humoral responses, and may inhibit thymus involution with age. In vitro MEL also increases T helper and NK cell activities, the production of interleukin 2 and interferon gamma, and the expression of interleukin 1 mRNA in human monocytes. In summary, most authors agree on an immuno-stimulating effect of MEL. These effects may occur via a direct action of MEL on its receptor since MEL-R have been identified in various tissues of the immune system, namely thymus, spleen, lymphocytes, and T helper cells.

In addition, MEL acting as a chronobiotic may be involved in the circadian organization of the immune system (the number and activity of lymphocytes T, B, and NK displaying a daily rhythmicity). It has also been

proposed that MEL may mediate seasonal changes in immune function, which is enhanced in short days with longer MEL peak duration (Nelson and Drazen, 2000).

4. Antioxidant/Antiaging Property of Melatonin. The publication of a revitalizing effect of MEL or of pineal youth transplants to old mice (Pierpaoli and Regelson, 1994) raised a general interest for MEL as an antiaging/antioxidant molecule. It was proposed that the lipophilic MEL diffuses into the cell cytosol and nucleus (Menendez-Pelaez and Reiter, 1993) to protect cytosolic and nuclear macromolecules from free radical cytotoxicity (see Reiter, 1995; Reiter et al., 2000b for reviews).

The use of oxygen in cell metabolism leads to the production of cytotoxic by-products that are reactive free radical species (superoxide anion radical, peroxynitrite anion, hydrogen peroxide, nitric oxide, and the highly toxic hydroxyl radical), which destroy macromolecules like DNA, lipids, and proteins leading to cell death via apoptosis. High doses of MEL (in the micromolar range) are reported to neutralize most of these cytotoxic molecules, but especially the hydroxyl radical, which is scavenged in vivo by MEL, producing cyclic 3-hydroxyMEL excreted in the urine. In addition, MEL is reported to stimulate the activity of various antioxidant enzymes, like superoxide dismutase or glutathione peroxidase, but inhibits the pro-oxidant enzyme nitric oxide synthetase.

Given that MEL could be a very powerful antioxidant molecule, that the production of MEL decreases with age (although this conception is now discussed, see Kennaway et al., 1999), and that the free radical effects are involved in the processes of aging and cancer, it has been suggested that maintaining MEL at a high level could slow age- and cancer-related alterations (Reiter, 1995; Reiter et al., 2000b). The anticarcinogenic effect of MEL is best described in vivo and in vitro on the estrogen-responsive mammary tumors (Tamarkin et al., 1981; Blask and Hill, 1986; Hill and Blask, 1988; Scott et al., 2001; Teplitzky et al., 2001; Kiefer et al., 2002). In vivo, there is an inverse correlation between the nocturnal level of plasma MEL and the number of estrogen receptors in patients with an estrogen-dependent cancer. In vitro, 1 to 100 nM MEL induces a 40 to 60% loss of MCF-7 cells (human breast tumoral cells). This cytotoxic effect of MEL is related to an apparent uncoupling of oxidative phosphorylation and leads to morphologic alteration and autophagocytosis. MEL also affects estrogen receptor transcriptional activity by regulating signal transduction pathways. In addition, MEL has been described as a potent supplement in the treatment or cotreatment of cancer: as an antioxidant, it may protect cell damage caused by carcinogens; as a chronobiotic, it may help determine optimum timing for carcinogen best efficiency; and it may act in synergy with the carcinogen retinoic acid to markedly reduce mammary tumor genesis in vivo.

It is noteworthy that most of these effects necessitate pharmacological doses of MEL (in the micromolar range) while plasma MEL concentrations are in the picomolar range. Recent studies, however, suggest that MEL could display antioxidant properties even at physiological levels (Pozo et al., 1994; Benot et al., 1999). Nevertheless, even if used at high doses, the therapeutic effect of MEL should not be neglected. Additionally, it is proposed that MEL could also serve to maintain synchronization of the main biological functions and prevent disintegration of the circadian oscillator in the course of aging (Armstrong and Redman, 1991).

D. Sites and Mechanisms of Action of Melatonin

The hormonal MEL message delivered by the pineal gland is distributed rapidly via the systemic circulation to all peripheral and central structures where MEL acts via specific binding sites (see Weaver et al., 1991; Masson-Pévet et al., 1994a, 1996; Morgan et al., 1994; Vanecek, 1998; von Gall et al., 2002b for reviews).

The localization and pharmacological characterization of the MEL binding sites were made possible in 1987 with the use of a radioiodinated MEL ligand (^{125}I-MEL, Vanecek et al., 1987). Two types of binding sites have been characterized: the high-affinity sites (with a constant of dissociation (K_D) between 20 and 200 pM), and the low-affinity sites (with a K_D value in the nanomolar range). Only the high-affinity sites have been characterized as receptors (MEL-R), and their genes cloned. Three types of high-affinity receptors have been characterized (see Reppert et al., 1996, for review; Dubocovich et al., 2001 for latest nomenclature): MT_1 (previously Mel_{1a}) present in all vertebrates, mainly in the brain; MT_2 (previously Mel_{1b}) present in all vertebrates, mainly in the retina; and Mel_{1c}, present in nonmammalian vertebrates. The low-affinity binding sites, MT_3, were recently described as the quinone reductase 2 enzyme (Nosjean et al., 2000).

The MT_1 receptor has seven transmembrane domains, specific to G-protein-coupled receptors, and are coupled negatively to the adenylate cyclase (AC) system. Their activation induces a decrease in forskolin-induced cAMP accumulation (Carlson et al., 1989; Morgan et al., 1989). This effect is generally mediated by a pertussis toxin-sensitive G-protein (G_i/G_o; Reppert et al., 1994). In the PT, MEL-Rs are coupled to two types of G-proteins, one sensitive to the pertussis toxin, the other to the cholera toxin. Other effects of MT_1 activation have also been reported on the intracellular concentrations of cGMP, diacylglycerol (DAG), inositol triphosphate (IP_3), or Ca^{2+}; on the activity of protein kinase Ca^{2+} and/or DAG-dependent (PKC); on the expression of *c-fos*; on the phosphorylation of cAMP responsive element (CRE)-binding protein (CREB); and on membrane potential.

Currently, about 110 cerebral structures express MEL binding sites. The number and nature of these structures display marked interspecific variations. In nearly

all mammals, the SCN mainly express MT_1 receptors with the exception of the mink and sheep. The PT is an endocrine structure characterized by a very high density of MT_1 receptors in all mammals except humans. The MT_2 receptor is present in the retina (Reppert et al., 1995) and possibly in the brain and SCN as well (Dubocovich et al., 1998; Isobe et al., 2001). In the SCN, MT_1 receptors would mediate the inhibitory effect on electrical activity, whereas the MT_2 receptor would mediate the phase-shifting effect of MEL. Notably, a nonsense mutation occurs in the MT2 coding gene in Siberian and Syrian hamsters, which disables the receptor (Weaver et al., 1996). MEL-Rs are present in the pineal gland of the newborn rat, become rare in 9-day-old rats, and are not detected in adults (Zitouni et al., 1995). MEL-Rs have also been characterized in many peripheral structures such as the Harderian gland, spleen, testis, ovary, vascular system, gut, smooth muscle, and some cells of the immune system (see Vanecek, 1998 for review).

E. Conclusion: Melatonin Is a Time-Giver Endocrine Messenger

MEL is a time-giver (*zeitgeber*) hormone. It is characterized by two rhythms of secretion: a 24-h rhythm with a nocturnal peak and an annual rhythm closely dependent on seasonal variations in the photoperiod. It is possible that most, if not all, functions attributed to MEL are related to the timing information it brings to different structures. Studies performed to understand the mechanisms of action of MEL in the regulation of some seasonal and circadian functions have demonstrated that the dynamic pattern of MEL secretion is fundamental for its time-giving function. The rhythmic pattern of MEL secretion is important because it brings to organisms information about time that allows them to adapt some of their physiological functions to the daily and seasonal variations of their environment. It is thus necessary to delineate the various processes and elements that regulate the rhythms of MEL synthesis and secretion to understand how environmental factors are transmitted to the whole organism.

III. Neural and Humoral Inputs to the Mammalian Pineal Gland

The mammalian pineal gland is a neuroendocrine structure targeted by numerous transmitters arriving via neural or endocrine pathways.

A. Structure and Ultrastructure of the Pineal Gland

The mammalian pineal structure and ultrastructure have been largely described in previous reviews (Vollrath, 1981; Pévet, 1983a). The pineal gland develops as an evagination of the diencephalic roof. In most mammals it forms a solid mass between the habenular and posterior commissures, but in rodents, whereas a deep and small part stays close to ventricle III, the major portion of the gland migrates in a dorso-caudal direction to form the superficial pineal, both parts being connected by the pineal stalk (see Fig. 1). The rodent superficial pineal gland is massively innervated and contains a dense network of blood vessels into which MEL is released. However, in the deep pineal gland, being made of functional pinealocytes that express the genes coding for the MEL-synthesizing enzyme with a day/night rhythm (Ribelayga et al., 1998a; Garidou et al., 2001), MEL could as well be directly released into the cerebrospinal fluid, as has been recently demonstrated in sheep (Tricoire et al., 2002). In the course of phylogenesis, the pineal gland has undergone marked transformations (Collin, 1971; Korf et al., 1998). Being made of true photoreceptors in lower vertebrates, in mammals it consists of neuroendocrine cells, the pinealocytes, with no direct light sensitivity but still expressing various photoreceptor markers (rhodopsin, *S*-antigen, recoverin, etc.). The mammalian pineal gland is a rather homogenous tissue containing mainly true pinealocytes (mono-, bi-, or tri-polar cells), few glial cells, phagocytic cells, and rare neurons.

B. Neural Inputs

The pineal gland is innervated with nervous fibers of various origins (Fig. 3). The main pathway consists of a complex route named the retino-hypothalamo-pineal pathway, ending with the sympathetic input to the pineal parenchyma. The pineal gland also receives neural inputs of central and parasympathetic origins. These pineal nerve endings contain a large variety of neurotransmitters.

1. Retino-Hypothalamo-Pineal Pathway. The rhythm in MEL synthesis depends essentially upon three interdependent factors: the endogenous circadian oscillator located in the SCN, the L/D cycle that synchronizes the endogenous oscillator, and the light that acutely inhibits nocturnal MEL synthesis. It is now well established that there exists a multi-synaptic neural pathway among the retina, SCN, and pineal gland. Various experiments (lesion, neuronal tracing) have allowed researchers to draw the general diagram of the main innervation of the mammalian pineal gland, especially in the rat (Moore and Klein, 1974; Klein and Moore, 1979; Moore, 1996; Larsen, 1999; Teclemariam-Mesbah et al., 1999).

a. The Retino-Hypothalamic Tract. Photic information is conveyed from the retina to the ventro-lateral zone of the SCN via the retino-hypothalamic tract (RHT). The light-sensitive cells forwarding the light/dark information do not appear to be the rod and cone photoreceptors (Lucas et al., 1999), but rather are a small subset of retinal ganglion cells containing the photopigment melanopsin (Moore et al., 1995; Berson et al., 2002; Hannibal et al., 2002; Hattar et al., 2002). The RHT neurotransmitters are mainly glutamate (Glu) (van den Pol, 1991; Ding et al., 1997) and pituitary

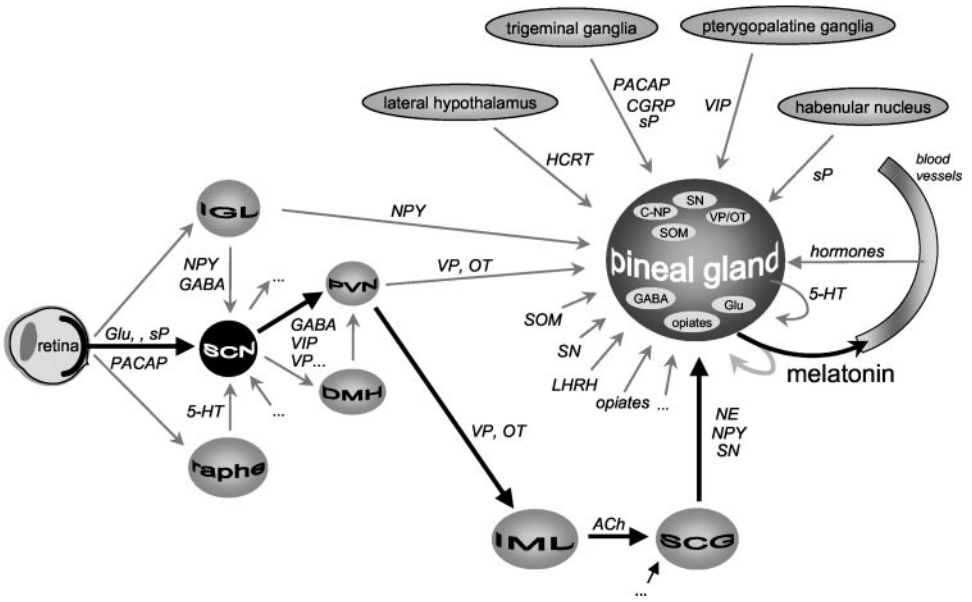

FIG. 3. Schematic representation of the various neural, endocrine, and paracrine inputs of the mammalian pineal gland. The main neural pathway, which transmits light information to the pineal gland, is shown with thick arrows. In addition, numerous other neural or endocrine inputs are known to reach the pineal gland. Note that there are interspecies differences in the density and origin of the afferent pineal nerve fibers and the nature of the different pineal transmitters.

adenylate cyclase activating peptide (PACAP) (Hannibal et al., 1997), but not substance P (sP), as previously thought (Takatsuji et al., 1991) mediating light signaling to the clock (see Hannibal, 2002 for review). Other inputs originating from the thalamic IGL, containing neuropeptide Y (NPY), enkephalin (Enk), and GABA (Card and Moore, 1982; Moore and Speh, 1993; Morin and Blanchard, 2001) and from the raphe nucleus, containing 5-HT (Moore et al., 1978) also carry photic and nonphotic information to the SCN (Mrosovsky, 1996).

b. The Hypothalamic Endogenous Circadian Oscillator. In mammals, several experiments have demonstrated the presence of an endogenous circadian oscillator in the SCN (see Ralph et al., 1990; Takahashi, 1995; Turek et al., 1995 for reviews) probably located in every SCN neuron showing an endogenous oscillation in firing rate (Welsh et al., 1995). This endogenous activity is higher during the subjective day and synchronized to exactly 24 h by the photic inputs. The cellular and molecular basis of this circadian oscillation and its synchronization are currently under active investigation. Several proteins (PER1–3, TIM, CLOCK, BMAL/MOP3, TAU/type Iε casein kinase, cryptochrome 1–2) work as transcription factors and enzymatic regulators in an autoregulatory transcriptional feedback loop constituting the core of the circadian pacemaker (see Whitmore et al., 1998, 2000; Dunlap, 1999; Ishida et al., 1999; Kume et al., 1999; King and Takahashi, 2000; Lowrey and Takahashi, 2000; van Esseveldt et al., 2000; Reppert and Weaver, 2001 for reviews). Other elements of the circadian clockwork are still being discovered. The central step in transducing the intracellular cycling of molecular clocks to the rhythm in spontaneous firing rate was recently demonstrated to involve L-type Ca^{2+} current (Pennartz et al., 2002). SCN neurons are mainly peptidergic cells containing vasoactive intestinal peptide (VIP), VP, gastrin-releasing peptide (GRP), and somatostatin (SOM), but also GABA (see Buijs et al., 1994, 1995; Inouye, 1996; van Esseveldt et al., 2000 for reviews). Some of the peptides in the SCN display daily and/or circadian rhythms in their synthesis and release, thus being putative clock outputs.

It is suggested that the hypothalamic clock could also be involved in the integration of seasonal information (see Pittendrigh and Daan, 1976; Illnerova and Vanecek, 1985, 1987; Pévet et al., 1996; Goldman, 2001; Hastings, 2001; Schwartz et al., 2001 for reviews). For example, FOS-light induction (Sumova et al., 1995; Vuillez et al., 1996; Jacob et al., 1997) and *Per1* gene expression (Messager et al., 1999b, 2000, 2001; Nuesslein-Hildesheim et al., 2000) in the SCN displays MEL-independent photoperiodic variations. In addition, the daily profile of *vp*-mRNA differs in long and short photoperiods (Jac et al., 2000). The integration of the photoperiod by the SCN has been proposed to involve two components (one recognizing variations of the dawn, the other of the dusk) with the increase (in the evening) and the diminution (in the morning) of MEL synthesis being regulated separately during photoperiod changes. The phase relationship between these two oscillator components would determine the duration of the nocturnal MEL peak (Illnerova and Vanecek, 1985, 1987). Recent observations in cultured SCN slices of Syrian hamsters have brought anatomical evidence for this concept (Jagota et al., 2000). However, an alternative view proposes that the photoperiod may be integrated into every SCN cell,

into the molecular mechanism of the circadian clock itself. By affecting the daily profile of the light-sensitive *Per* expression (long under LP, short under SP), photoperiod may, in turn, affect the kinetics of the expression of the clock proteins and consequently the expression of all the clock-regulated genes (see Hastings, 2001 for review). Although it has been demonstrated that photoperiod clearly regulates the daily profile of *Per1* (Messager et al., 2000) and PER1 (Nuesslein-Hildesheim et al., 2000) in the SCN, the link between changes in the clock-gene expression profile and SCN outputs remains to be established.

c. Suprachiasmatic Nucleus of the Hypothalamus Outputs to the Pineal Gland. Many studies seek to elucidate how the temporal information generated by the SCN is transmitted to the organism to regulate many rhythmic physiological and behavioral functions (see Buijs, 1996; Buijs and Kalsbeek, 2001; Kalsbeek and Buijs, 2002 for reviews). It is generally considered that the ventro-lateral part of the SCN is the clock input area for the synchronizing events while the dorso-median part contains the oscillator and the output of the timing information. Actually, various SCN neurons project mainly to different hypothalamic structures to transmit the timing information to different functional axes, especially the hypothalamo-pituitary-adrenal axis (rhythmic secretion of corticosterone) and the hypothalamo-pineal axis (rhythmic secretion of MEL). Recently, the link between the SCN output and the circadian rhythm in locomotor activity was proposed to be the transforming growth factor α acting on the hypothalamic subparaventricular zone (Kramer et al., 2001). In addition, the SCN could regulate peripheral endocrine organs via the autonomic nervous system (Buijs et al., 1999, 2001; Kalsbeek et al., 2000a; La Fleur et al., 2000). The increasing use of cDNA microarrays will help to identify new clock-controlled genes in various tissues (Akhtar et al., 2002; Duffield et al., 2002; Humphries et al., 2002).

In the rat, the SCN neurotransmitters involved in the clock output would be essentially VP and GABA (Moore and Speh, 1993; Buijs et al., 1994; Kalsbeek et al., 1995; 1996a). VP appears to be a good clock-controlled transmitter since 1) it displays a circadian rhythm of synthesis and release (Reppert, 1985; Murakami et al., 1991; Kalsbeek et al., 1995; Watanabe et al., 2000); 2) its gene promoter, containing an "E-box," is under the direct control of the clock genes (Jin et al., 1999); and 3) it acts on the dorsomedial hypothalamus to control the circadian rhythm of corticosterone synthesis and release (Kalsbeek et al., 1996b). In addition, VIP (Teclemariam-Mesbah et al., 1997a), glutamate (Cui et al., 2001), or another unknown diffusible substance (Silver et al., 1996; Allen et al., 2001) may also be non-neural outputs of the molecular clock.

As far as the regulation of MEL synthesis is concerned, the hypothalamic paraventricular nuclei (PVN) are an essential relay between the SCN and the pineal gland. PVN lesions abolish the rhythm of MEL synthesis in the pineal gland (Klein et al., 1983), PVN neurons respond to an electrical stimulation of SCN cells (Hermes et al., 1997), VIP or VP infusion in the PVN elevates pineal melatonin release (Kalsbeek et al., 1993), and retrograde labeling from the pineal gland is seen in the PVN (Larsen, 1999; Teclemariam-Mesbah et al., 1999). GABA appears to be involved in transmitting signals from the SCN to the PVN since infusion of a GABA antagonist during the subjective day in the PVN area stimulates MEL synthesis, whereas infusion of GABA during the night inhibits nighttime MEL secretion (Kalsbeek et al., 1996a, 1999, 2000b). SCN lesions abolish the daily rhythm of MEL synthesis but keep MEL at a level intermediate between daytime and nighttime values. These data indicate that the SCN is a daytime inhibitor (via GABA) of the PVN stimulation of MEL synthesis, and is probably also a nighttime stimulator (Kalsbeek et al., 2000b).

The dorsal and lateral parvocellular neurons of the PVN, containing oxytocin (OT) and VP, reach the intermediolateral cells (IML) of the upper three segments of the spinal cord (Gilbey et al., 1982; Yamashita et al., 1984; Cechetto and Sapper, 1988; Teclemariam-Mesbah et al., 1997b; Larsen, 1999). Diurnal inhibition of pineal gland activity could also take place at this level since 1) infusion of VP and especially OT in the IML inhibits the electrical activity of the preganglionic neurons of the spinal cord (Gilbey et al., 1982); and 2) inhibition of MEL synthesis following PVN electrical stimulation (Reuss et al., 1985; Olcese et al., 1987) is abolished in VP-deficient Brattleboro rats (Reuss et al., 1990). The IML neurons innervate the rostral pole of the superior cervical ganglion (SCG) neurons that project to the pineal gland (Strack et al., 1988; Reuss et al., 1989). This last step is excitatory since electrical SCG stimulation increases MEL release (Bowers and Zigmond, 1980). ACh is the main neurotransmitter released in the SCG (Kasa et al., 1991), but other neurotransmitters, especially SOM, VIP, histidine isoleucine peptide (PHI), and calcitonin gene-related peptide (CGRP) are potential candidates in the transmission of information to the SCG. Approximately 0.5 to 1% of SCG neurons project to the pineal gland (Bowers et al., 1984; Larsen, 1999).

The mammalian pineal gland is characterized by a very dense sympathetic innervation (see Kappers, 1960; Korf, 1996; Møller, 1999; for reviews). The first demonstration of the presence of neurotransmitters in the rat pineal gland was made using the technique of Falck et al. (1962), which showed the presence of NE in the sympathetic fibers of the pineal gland. In the rat (Zhang et al., 1991) and sheep (Cozzi et al., 1992) pineal gland most of the tyrosine hydroxylase (TH; the rate-limiting enzyme for NE synthesis) immunoreactive fibers disappear after the SCG removal (SCGx). The remaining fibers could originate from central neurons. The sympathetic fibers of the pineal gland also contain DA, 5-HT,

VIP, and especially NPY. These fibers enter the distal part of the pineal via the conarian nerves (*nervi conarii*). Inside the pineal gland they follow the vascular system. In some species, the fibers enter the gland parenchyma and end between the pinealocytes. They never make true synapses with the pinealocytes, but synaptic-like junctions between NAergic endings and pinealocytes are sometimes observed (Huang and Lin, 1984; Masson-Pévet et al., 1987a). In some rodent species the sympathetic fibers spread out of the pineal gland toward the habenular nuclei (Korf et al., 1990). In some species, a few pinealocytes of the deep pineal gland project to neighboring central structures (the habenular nucleus, the pretectal areas) where they make synapses (Korf et al., 1986, 1990; Sato et al., 1991). The putative transmitters involved have not been identified, but this observation suggests that the pineal gland could exert its influence by a neuronal pathway in addition to the MEL endocrine pathway. In the rat, the pinealocytes do not show such projections (Korf et al., 1986; Ribelayga et al., 1998a).

2. Central Pathways. In 1975–1985, numerous studies using electrophysiological and neuroanatomical techniques demonstrated that the mammalian pinea Møller l gland receives a diversified central innervation although it is less dense than the sympathetic innervation. These observations have led to the hypothesis that various central structures play a physiological role in the regulation of the metabolic activity of the mammalian pineal gland (see Korf and Møller, 1984, 1985; Korf, 1996; Møller et al., 1996, Møller, 1999; Moller and Baeres, 2002 for reviews).

The early ultrastructural observations had already suggested the presence of extra-sympathetic fibers since 1) the pineal gland exhibits synaptic buttons containing large (100 nm) granular vesicles (peptidergic type) or small (40–60 nm) clear vesicles (cholinergic type); 2) myelinated fibers observed in the pineal gland are still preserved after SCGx (Lin et al., 1975; Schneider et al., 1981; Møller and Korf, 1983a); and 3) lesions of the habenular area induces the degeneration of fibers and nerve endings in the rodent pineal gland (David and Herbert, 1973; Ronnekleiv and Møller, 1979; Møller and Korf, 1983a). Use of the horseradish peroxidase (HRP) tracing technique has confirmed the existence of neural connections between the brain and pineal gland in several rodent species. When tracer was injected into the pineal gland, HRP-positive fibers were observed in the proximal part of the gland continuing either via the posterior commissure or via the habenular commissure. HRP-positive neurons were observed in the habenular nuclei, the posterior commissure nuclei, the PVN and, in some cases, the IGL (Korf and Wagner, 1980; Guérillot et al., 1982; Møller and Korf, 1983b). These initial observations were confirmed by anterograde tracing from the PVN (Møller et al., 1990a; Larsen et al., 1991), the lateral hypothalamus (Fink-Jensen and Møller, 1990), the habenular nuclei, and the IGL (Reuss and Møller, 1986; Mikkelsen and Møller, 1990; Mikkelsen et al., 1991) showing positive fibers in the proximal part of the pineal gland. The neurotransmitters observed in these central fibers are mainly neuropeptides, especially VP and OT (PVN: Buijs and Pévet, 1980), sP (habenular nuclei: Ronnekleiv and Kelly, 1984), and NPY (IGL: Mikkelsen et al., 1991). In addition, histaminergic fibers originating in the tuberomammillary nucleus (Mikkelsen et al., 1992), 5-HTergic fibers originating in the dorsal raphe (Leander et al., 1998), and hypocretin (HCRT)-containing fibers originating in the lateral hypothalamus (Mikkelsen et al., 2001) were also demonstrated in the rodent pineal gland.

The use of electrophysiological techniques has also confirmed the existence of pineal fibers of central origin. Stimulation of central structures such as the PVN, lateral hypothalamus, amygdala, hippocampus, and especially the habenular nuclei induced an electrophysiological response of the pinealocytes (Dafny, 1977; Ronnekleiv et al., 1980; Semm, 1981, 1983; Reuss et al., 1984, 1985). Furthermore, with the use of this technique it was reported that light could be transmitted to the pineal gland via the sympathetic system with a long latency, but also via other central pathways with a shorter latency (Dafny, 1980).

In summary, these studies have demonstrated that, in addition to the dense sympathetic innervation, other fibers of a lower density, originating from various central structures (especially the habenular nuclei, PVN, IGL, dorsal raphe, and lateral hypothalamus) innervate the rodent pineal gland. Central fibers arrive and terminate mostly in the proximal part of the pineal gland. This does not exclude a physiological effect of these central inputs because 1) electrophysiological and biochemical connections occur between the pinealocytes (Reuss et al., 1984, Saez et al., 1994) and 2) the stalk and the deep part of the rodent pineal gland possess true pinealocytes that contain the enzymes of MEL synthesis (Ribelayga et al., 1998a; Garidou et al., 2001). In nonrodents, where the whole pineal gland is located close to the third ventricle, the central innervation may be denser and thereby functionally more important (see Møller, 1999 for review). The existence of neural connections among the PVN, IGL, and raphe on the one hand, and the pineal gland on the other hand, is of particular interest because these three structures are involved in the regulation of hypothalamic clock activity. In addition, the activity of these three structures is directly (IGL, raphe) or indirectly (PVN) modulated by light. Since short light exposure at night induces a very rapid diminution of MEL synthesis and release (Klein and Weller, 1972; Illnerova et al., 1979; Drijfhout et al., 1996c), it is possible that the central pathway involved in this rapid light inhibition (Dafny, 1980) passes through one and/or another of these structures. The results of selective lesion experiments have suggested

that the central IGL-pineal pathway could be involved in the rapid inhibitory effect of a light flash on the metabolic activity of the pineal gland (Cipolla-Neto et al., 1995; Bartol et al., 1997).

3. Parasympathetic Pathways. The presence of a parasympathetic innervation of the pineal gland has long been debated (see Phansuwan-Pujito et al., 1999, for review). However, localization of pinealopetal fibers originating in the pterygopalatine and the otic ganglia (Shiotani et al., 1986; Møller and Liu, 1999) together with the demonstration of pineal cholinergic fibers in the rat (Eranko et al., 1970), ferret (David and Herbert, 1973), rabbit (Romijn, 1973), monkey (David and Kumar, 1978), and cow (Phansuwan-Pujito et al., 1990, 1991b) have demonstrated the occurrence of a parasympathetic input to the pineal gland. Besides ACh, VIPergic fibers originating from parasympathetic ganglia have also been observed in the rodent pineal gland (Shiotani et al., 1986; Møller and Liu, 1999). In addition, demonstration of receptors and biochemical effects of cholinergic and VIPergic ligands in the pineal gland (see *Sections VI.A.* and *VI.B.*) confirm the existence of parasympathetic control of pineal activity.

4. Pathways from Other Neural Structures Retrograde tracing studies have demonstrated that the trigeminal ganglia project directly to the rodent pineal gland (Shiotani et al., 1986; Reuss et al., 1992a; Møller and Liu, 1999; Reuss, 1999). These fibers contain sP, CGRP, and PACAP. The trigeminal input to the pineal gland is interesting because to date this ganglion has only been considered a sensory ganglion.

C. Endocrine Inputs

Because the pineal gland is outside the blood-brain barrier in most species, substances secreted into the bloodstream may affect pineal activity as long as receptors for those substances are present in the pineal gland (see Moller and Baeres, 2002 for review). For example, this has been shown for the pituitary peptides and gonadal hormones. Radioactive labeled peptides such as luteinizing hormone-releasing hormone (LHRH; Redding and Schally, 1973), melanin-stimulating hormone (Kastin et al., 1976), and delta-sleep inducing peptide (DSIP; Graf and Kastin, 1984) injected into the bloodstream accumulate in the pineal gland. VP and OT, released into the circulation during osmotic regulation or during parturition and lactation, may also act on pineal activity. VP, for example, concentrates in the pineal gland (Zlokovic et al., 1991). Other circulating peptides such as natriuretic factors may also alter pineal activity since in vitro effects of these peptides have been observed. Some gonadal steroids also concentrate in the pineal gland, where they alter its activity (Nagle et al., 1972, 1974).

D. Paracrine Inputs

In the pineal gland MEL is synthesized from intracellular 5-HT, then released into the bloodstream. It has been reported that 5-HT (see *Section VI.B.1.*) and MEL (see *Section II.C.1.*) display additional autocrine/paracrine effects. Pineal cells also contain GABA (15% of bovine pineal cells: Rosenstein et al., 1989b), Glu (McNulty et al., 1992), aspartate (Imai et al., 1995), and taurine (LaBella et al., 1968), which are able to alter the metabolic activity of the pineal gland (see *Section VI.B.*). Intrapineal neurons immunopositive for acetylcholinesterase have been identified in the pineal gland of several mammalian species (Romijn, 1975; Phansuwan-Pujito et al., 1999). Growth factors are present in the pineal gland (Garcia-Maurino et al., 1992) where they favor neurite development of the pinealocytes (McNulty et al., 1993).

A particularity of the mammalian pineal gland is the ability to synthesize various peptides that are able to alter its metabolic activity. However, the original data showing this were the subject of discussion because the immunocytochemistry technique used poorly specific antibodies (see Pévet et al., 1980b for discussion). Later on, the techniques of in situ hybridization (ISH) and reverse transcription followed by polymerization chain reaction (RT-PCR) have confirmed peptide synthesis in mammalian pineal cells. Cells containing Enk have been characterized in the rodent pineal gland (Schröder et al., 1988; Aloyo, 1991; Coto-Montes et al., 1994). In the European hamster, Enk-containing cells display synaptic-like connections with other pineal cells (Coto-Montes et al., 1994), suggesting a paracrine function of this peptide. In the rat, combined studies of ISH for preproEnk and immunocytochemistry for 5-HT have shown that cells expressing the peptide (approximately 7%) are not pinealocytes, but rather glial cells (Wang et al., 1996). Pineal cells also contain LHRH (rat, Pévet et al., 1980b), SOM (rat, Pévet et al., 1980b; Møller et al., 1995), sP (cotton rat, Matsushima et al., 1994), C-type natriuretic peptide (CNP) (cytoplasmic vesicles of bovine pineal cells, Middendorff et al., 1996). The pineal gland of the Syrian hamster, but not the rat, displays a few cells containing secretoneurin (SN) (Simonneaux et al., 1997a). The presence of VP in pineal cells is a matter of discussion since the mRNA coding for VP has been detected in the pineal gland of rat (Lepetit et al., 1993), sheep (Matthews et al., 1993), and cow (Olcese et al., 1993), but no VP-IR cells have yet been observed. This suggests that VP is synthesized in low amounts in pineal cells, the mRNA is present but not translated, or the mRNA is present in VPergic neural fibers but not in pineal cells. In contrast to VP, the presence of a few OT-containing neuron-like cells in the bovine pineal gland has been demonstrated both by ISH and immunocytochemistry (Badiu et al., 2001). Several studies have shown the presence of high concentrations of type α melanin-stimulating hormone (αMSH) in the pineal

gland of several species (Oliver and Porter, 1978; Vaudry et al., 1978; Pévet et al., 1980b; Schröder et al., 1988). The majority of peptide-containing cells are neuron-like or modified pinealocytes displaying synaptic contacts with the true pinealocytes. It is noteworthy, however, that the density of these peptidergic cells is usually very low. These active substances synthesized in the pineal gland may display auto/paracrine effects in the pineal gland because most of them are able to modify pineal metabolism in vitro. It is evident that some of these substances, in addition to MEL, could have an endocrine function. However, currently there are no sufficient data on this subject.

The observation of protein-containing granular vesicles in the pineal gland of some species and of rare exocytosis (Masson-Pévet et al., 1987b) has led to the search for pineal-specific peptides displaying pro or antigonadotropic effects. These studies have brought few satisfactory results (see Pévet, 1981, 1983b; Vaughan, 1984 for reviews). A decapeptide isolated from the bovine pineal gland has been characterized for its inhibitory effect on prolactin secretion and luteinizing hormone pulses (Benson and Ebels, 1994; Benson et al., 1996).

E. Conclusion: The Pineal Gland Is a Junction of Various Neural Inputs

The metabolic activity of the mammalian pineal gland is mainly under the control of the hypothalamic clock, its temporal message being delivered to the pineal gland by a polysynaptic pathway ending with sympathetic fibers. However, "the various neuroanatomical and immunocytochemical data now have profoundly changed the former concept that the mammalian pineal gland is solely innervated by the sympathetic nervous system-"(Møller, 1999). Actually, the pineal gland is the target of several (neuro)transmitters of various origins (Fig. 3). These findings have led to numerous biochemical studies to understand how, besides NE (see *Section V.*), these other pineal transmitters regulate the synthesis of MEL (see *Sections VI.A.* and *VI.B.*).

IV. Indoleamine Metabolism in the Mammalian Pineal Gland

The metabolic activity of the pineal gland has already been reviewed in earlier papers (Klein et al., 1981a; Bittman, 1984; King and Steinlechner, 1985; Klein, 1985; Sugden, 1989).

A. Indoleamine Metabolic Pathways

Tryptophan (Trp), taken up from the bloodstream, is the synthetic precursor of all the pineal 5-methoxyindoles (Fig. 4). Trp is metabolized into 5-hydroxy-Trp (5-HTP) in the pineal mitochondria by Trp-hydroxylase (L-Trp tetrahydropteridin:oxygen oxidoreductase; EC 1.14.16.4, TPOH) (Lovenberg et al., 1967), which is then converted into 5-HT in the pineal cytosol by an aromatic amino acid decarboxylase (EC 4.1.1.28, AAAD) (Lovenberg et al., 1962; Snyder and Axelrod, 1964). A fraction of 5-HTP may be methylated into 5-methoxytryptophan (Balemans et al., 1978a,b). 5-HT is the initial substrate of three different synthetic pathways:

1. 5-HT can be directly *O*-methylated by hydroxyindole-*O*-methyltransferase (*S*-adenosyl L-methionine: hydroxyindole-*O*-methyltransferase; EC 2.1.1.4; HIOMT) (Axelrod and Weissbach, 1960) into 5-methoxytryptamine (Axelrod and Weissbach, 1961);
2. 5-HT can be deaminated by monoamine oxidase (amine:oxygen oxidoreductase; EC 1.4.3.4; MAO) into 5-hydroxyindole-acetaldehyde (5-HIAL). This compound is then either successively oxidized into 5-hydroxyindole acetic acid (5-HIAA) by an aldehyde dehydrogenase (aldehyde:NAD^+ oxidoreductase; EC 1.2.1.3) then *O*-methylated by HIOMT to form 5-methoxyindole acetic acid (5-MIAA), or successively reduced into 5-hydroxytryptophol (5-HL) by an alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase; EC 1.1.1.1) then *O*-methylated by HIOMT to form 5-methoxytryptophol (5-ML);
3. The physiologically most important metabolic pathway of 5-HT leads to the synthesis of MEL (Weissbach et al., 1960; Axelrod et al., 1969). 5-HT is first acetylated by arylalkylamine-*N*-acetyltransferase (acetyl CoA:arylalkylamine-*N*-acetyltransferase, EC 2.3.1.37; AA-NAT) into *N*-acetylserotonin (NAS) (Weissbach et al., 1960; Voisin et al., 1984), then *O*-methylated by HIOMT to form MEL (Axelrod and Weissbach, 1960). In the rat, the quantity of MEL in the pineal gland increases from approximately 100 to 200 pg (0.43 to 0.86 pmol) per gland during the daytime to 1 to 2 ng (4.3 to 8.6 pmol) per gland at night. This gives plasma concentrations of 10 to 20 pg/ml (43 to 86 pM) and 80 to 100 pg/ml (344 to 430 pM), respectively. MEL, being a lipophilic molecule, it is not stored but directly released by diffusion out of the pineal gland. The half-life of MEL is approximately 20 min in the bloodstream (Gibbs and Vriend, 1981). In the rat, it is rapidly degraded in the liver into 6-hydroxy-MEL via cytochrome P450 (Skene et al., 2001), then sulfated into 6-sulfatoxy-MEL and eliminated in the urine (Kopin et al., 1961; Kveder and McIsaac, 1961). In the mouse, in contrast, melatonin is metabolized into 6-glucuronylmelatonin (Kennaway et al., 2002). Measurement of MEL in the pineal gland or in the plasma at any given time tightly reflects its synthesis (Illnerova et al., 1978). These characteristics give MEL a highly dynamic resolution that is essential for its time-giving properties.

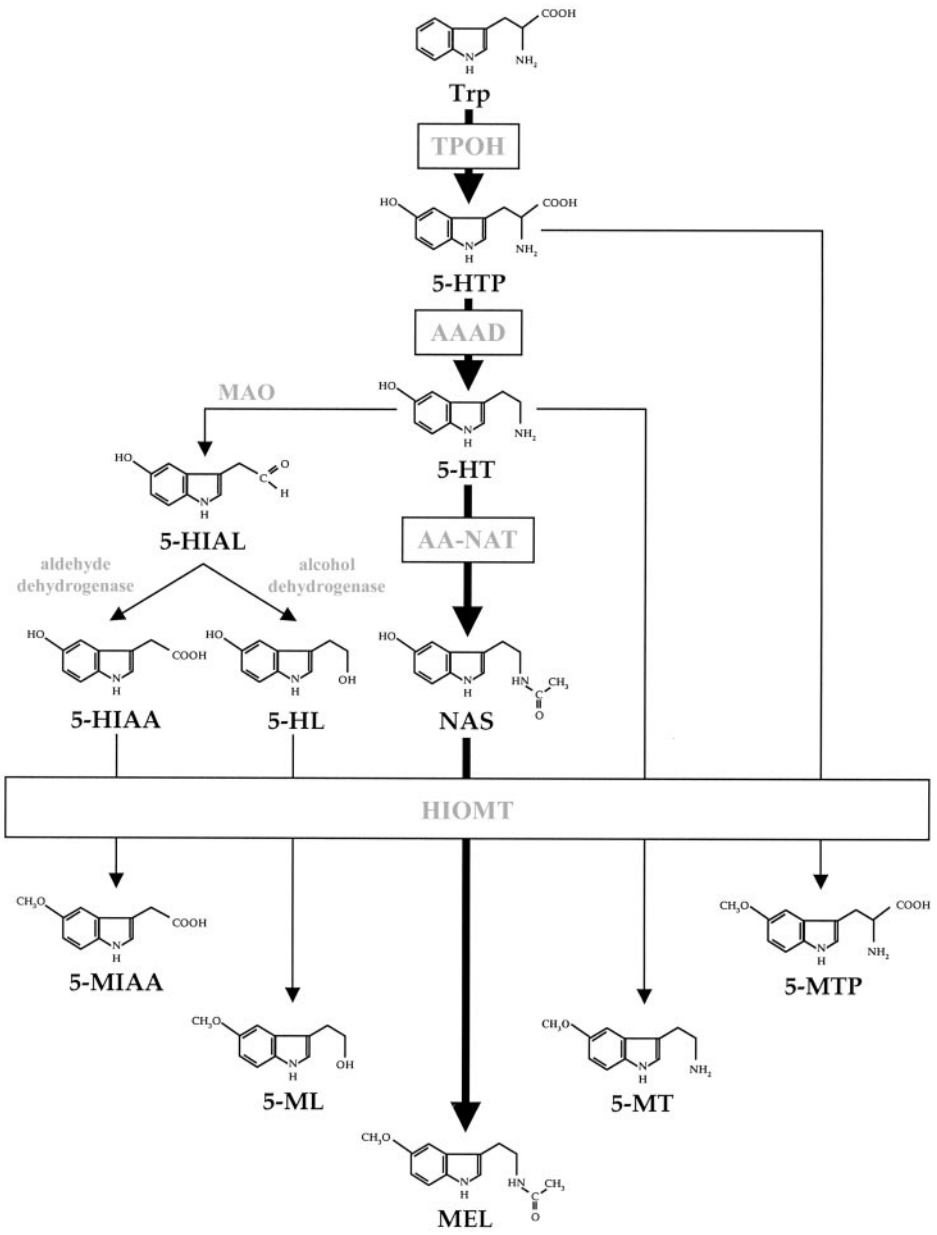

FIG. 4. Metabolism of indoleamines in the mammalian pineal gland. The essential amino acid tryptophan (TRP) is the initial substrate for five different synthetic pathways, of which the MEL metabolic route (thick arrows) is physiologically the most important. Note the central role of HIOMT.

B. Tryptophan Hydroxylase

The rat pineal *Tpoh* gene codes for two transcripts of 1.8 and 4 kb (Darmon et al., 1988). They contain the same coding sequence, but differ by the length of the 3′ noncoding region. The promoter region of the *Tpoh* gene contains not a canonical CRE motif (Stoll and Goldman, 1991; Boularand et al., 1995), but an inverted CCAAT box and a GC-rich region that bind the transcription factors NF-Y and Sp1, both being essential for *Tpoh* gene transcription at the basal level and following cAMP treatment (Côté et al., 2002).

The TPOH protein, whose presence in the rat pineal gland was demonstrated by Lovenberg et al. in 1967, displays a rather short half-life, approximately 75 min (Sitaram and Lees, 1978). It may be phosphorylated by the cAMP-dependent protein kinase (PKA) (Ehret et al., 1991; Johansen et al., 1995, 1996), a Ca^{2+}/calmodulin (CaM)-dependent protein kinase (PKCa^{2+}/CaM) (Ehret et al., 1989) and PKC (Ehret, 1994). In the pineal gland, it has been demonstrated that stimulation of PKA induces TPOH activation. TPOH activity measured in pineal homogenates at optimal temperature (37°C) and pH (7.5) and with saturating substrate concentrations, varies between 6 (day) and 12 (night) nmol/h/gland (Ehret et al., 1991). *Para*-chlorophenylalanine (*p*-CPA) is a selective and powerful inhibitor of TPOH activity (Deguchi and Barchas, 1972a,b).

Tpoh gene expression and enzyme activity display daily variations (Fig. 5). Their levels are already high during the day and increase further during the night by 20% (Besançon et al., 1996) and 100% (Shibuya et al., 1978; Ehret et al., 1991), respectively. The nocturnal increase in TPOH activity is more sensitive to the action of a protein synthesis inhibitor (cycloheximide) than to that of a transcription inhibitor (actinomycin D), suggesting that the increase results mainly from post-transcriptional/post-translational mechanisms (Sitaram and Lees, 1978, 1984; Ehret et al., 1991; Sun et al., 2002).

5-HT concentrations display a daily rhythm in the rat pineal gland, with high values during the day (150 to 250 pmol/gland, approximately 0.5 mM) and lower values during the night (25 to 50 pmol per gland) (Snyder et al., 1965b, 1967; Quay, 1974). These variations are opposite to that of MEL, and are therefore supposed to reflect the nocturnal use of 5-HT to synthesize MEL (Mefford et al., 1983). Recent results, however, have shown that nocturnal synthesis and release of 5-HT is more complex and is required for maximal NAergic stimulation of MEL synthesis (Miguez et al., 1997; Sun et al., 2002; see *Section VI.B.1.*).

C. Aromatic Amino Acid Decarboxylase

AAAD is an enzyme not specific to the pineal gland. It is present in large quantities in the cytosolic fraction of the pinealocytes (Snyder and Axelrod, 1964) and it is not a limiting factor for the synthesis of 5-HT (see King and Steinlechner, 1985 for review).

D. Monoamine Oxidase

MAO activity is detectable in the pinealocytes and in the NAergic nerve endings (Yang et al., 1972). This differential distribution reflects two types of MAO: type A in the nerve terminals and type B in the pinealocytes. These two types of MAO are characterized by different biochemical properties and sensitivity to inhibitors. It appears that MAO A is mainly involved in 5-HT oxidation (King and Steinlechner, 1985; Masson-Pévet and Pévet, 1989). Consequently, it has been proposed that 5-HT exits the pinealocytes to be oxidized in the NAergic nerve terminals and then returns to the pinealocytes. MAO activity displays day/night variation with higher values during the day (see King and Steinlechner, 1985 for review).

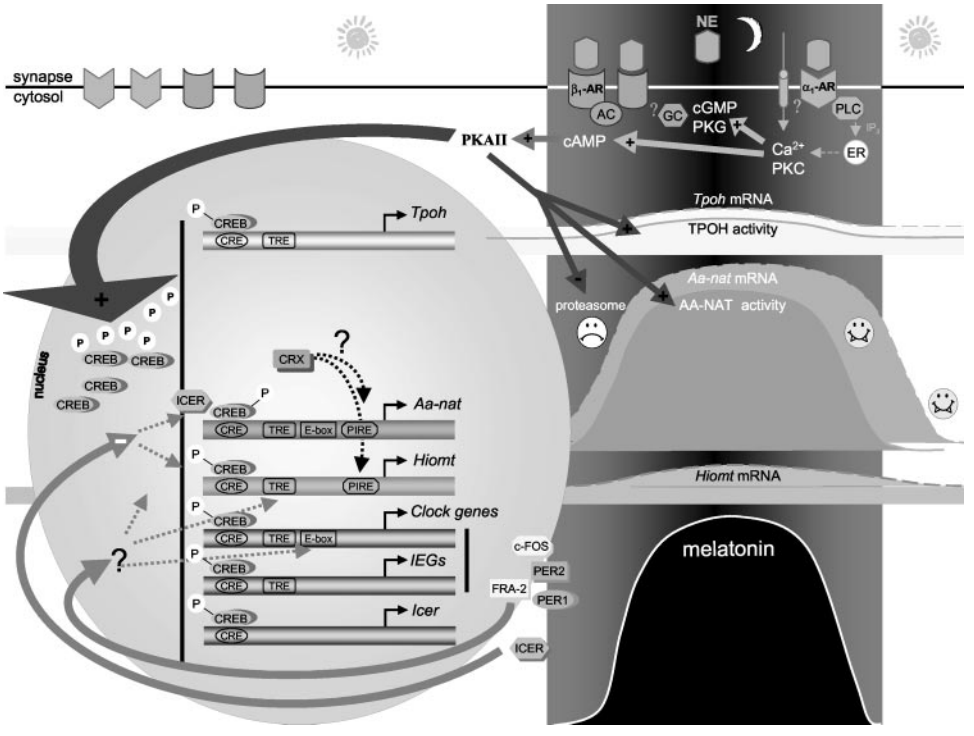

FIG. 5. Intracellular effects following nocturnal adrenergic stimulation of rat pinealocytes. In in vivo conditions, NE is released at night and activates two types of postsynaptic adrenergic receptors. Activation of the β_1-type AR is required and sufficient to trigger MEL synthesis, and results in a dramatic accumulation of the cyclic nucleotides cAMP and cGMP, although only cAMP is involved in the stimulation of MEL synthesis. Activation of the β_1-type AR has no effect per se on MEL synthesis but substantially potentiates β_1-AR activation through Ca^{2+} mobilization and PKC activation. The marked increase in cAMP content induces two independent mechanisms: 1) cAMP activates PKA, whose catalytic subunit translocates into the nucleus and in turn phosphorylates CREB. This event switches on the expression of different genes: those coding for the enzymes involved in MEL synthesis and different classes of transcription factors, the repressor ICER, the clock proteins, and different IEGs. In the rat, the nocturnal increase in MEL synthesis results primarily from a dramatic stimulation of *Aa-nat* gene expression and consequently of AA-NAT activity; 2) cAMP allows the accumulation of active molecules of AA-NAT by inducing PKA-dependent phosphorylation of AA-NAT, which interacts with the chaperone protein 14-3-3, and protects AA-NAT from lysis by the cytosolic proteasome. In addition, PKA increases TPOH activity during the night. Toward the end of the night the rapid clock-controlled decrease in adrenergic stimulation quickly lowers the cAMP intracellular content. Loss of the cAMP/PKA protective effect on the AA-NAT molecules results in their lysis by cytosolic proteasome, and consequently leads to a rapid decrease in MEL synthesis independent of *Aa-nat* mRNA.

E. Alcohol and Aldehyde Dehydrogenases

Neither alcohol nor aldehyde dehydrogenase is saturated by 5-HIAL. Pineal concentrations of 5-HIAA and 5-HL vary similarly with those of 5-HT. The 5-HIAA/5-HL ratio is around 1:6, and is probably related to the lower affinity of alcohol dehydrogenase for its substrate (see King and Steinlechner, 1985 for review).

F. Arylalkylamine-N-Acetyltransferase

The AA-NAT enzyme, catalyzing *N*-acetylation of 5-HT, was first identified as the arylamine-*N*-acetyltransferase (EC 2.3.1.5; NAT) (Weissbach et al., 1960). In reality, two types of *N*-acetyltransferase are present in the pineal gland: the arylamine- and the arylalkylamine-*N*-acetyltransferase named after their best substrates (Voisin et al., 1984). Because the affinity of 5-HT is much higher for AA-NAT than for A-NAT, only the former enzyme is involved in the rhythmic synthesis of MEL. Its activity displays marked day/night variation, especially in the rat (Klein and Weller, 1970).

The cDNA coding for *Aa-nat* has been recently isolated, first in the rat (Borjigin et al., 1995; Roseboom et al., 1996), then in sheep (Coon et al., 1995), human (Coon et al., 1996), monkey (Klein et al., 1997; Coon et al., 2002), mouse (Roseboom et al., 1998), cow (Craft et al., 1999), Syrian hamster (Gauer et al., 1999), and rat grass (Garidou et al., 2002) with few interspecies differences in the *Aa-nat* gene sequence (see Klein et al., 1997 for review). The *Aa-nat* gene is located on chromosome 11, in position E1.3–2.3, in the mouse, on chromosome 10q32.3 in the rat (Yoshimura et al., 1997), and on chromosome 17q25 in the human (Coon et al., 1996). It is organized into three introns and four exons. In mammals, the *Aa-nat* gene codes for only one transcript whose size varies between 1.0 and 1.7 kb according to species. In most species, it may be expressed in several tissues: 1) pineal and retina with a high level of expression; and 2) different nervous tissues (like PT, SCN, hippocampus), and peripheral structures (mainly testis and ovaries) with a much lower level of expression (see Borjigin et al., 1995; Coon et al., 1996; Klein et al., 1997; Fleming et al., 1999; Hamada et al., 1999; Uz and Manev, 1999 for review). Besides the pineal gland and retina, which synthesize MEL, whether AA-NAT regulates local synthesis of 5-HT or NAS in other structures remains to be established. In the rat, the promoter region of the *Aa-nat* gene has been studied (Fig. 5). It contains one CRE-like sequence (differing by one base from the perfect CRE sequence and named natCRE), an inverted CCAAT box and an activating protein-1 (AP-1) site (Baler et al., 1997). The natCRE site is capable of binding the phosphorylated form of CREB (P-CREB), whereas CCAAT box activation by specific binding proteins (CATBP) also appears necessary for large activation of *Aa-nat*. cAMP-induced *Aa-nat* gene transcription therefore requires activation of a CRE-CCAAT complex. A perfect CRE site has also been recently characterized in the promoter region of the *Aa-nat* gene, and appears critical to achieve full stimulation of *Aa-nat* gene expression (Burke et al., 1999). Another *cis*-DNA sequence named E-box (able to mediate transcriptional up regulation via the action of the BMAL1/CLOCK heterodimer) has been identified in the first intron of the rat *Aa-nat* gene (Chen and Baler, 2000). However, transfection of pinealocytes with *Bmal1*/*Clock* was unable to induce *Aa-nat* transcription, whereas the same kind of transfection in retinal cells led to activation of *Aa-nat* gene expression (Chen and Baler, 2000). In the chicken pineal gland, which in contrast to the mammalian pineal gland contains an endogenous oscillator, the *Aa-nat* E-box binds the BMAL1/CLOCK heterodimer that enhances transcription (Chong et al., 2000). These data suggest that 1) in the rat the regulation of *Aa-nat* gene expression is radically different in a *slave* (the pineal) compared to a *master* oscillator (the retina where *Aa-nat* gene is a possible output of the clock molecular loop), and 2) chicken *Aa-nat* transcriptional activation by clock protein heterodimers is critical for rhythmic expression of the enzyme activity. Finally, the pineal *Aa-nat* gene promoter contains a pineal regulatory element (PIRE) that binds the transcription factor cone-rod homeobox (CRX) that is exclusively expressed in photoreceptors and pinealocytes (Li et al., 1998).

AA-NAT is an approximately 23-kDa soluble cytosolic protein. It displays an N-terminal area involved in the binding of the arylalkylamines and a C-terminal area with two well preserved motifs, named A and B, which are supposed to bind the cofactor acetyl coenzyme A (see Klein et al., 1997, 2002 for reviews). According to the deduced amino acid sequence, homology with the human AA-NAT is 97% in the monkey, 84% in the sheep, and 90% in the rat. Several putative sites of phosphorylation (for the PKA, the PKC, and the casein kinase of type II) are present and well preserved across species (Klein et al., 1997). The rat AA-NAT proteic structure is globular, made of eight β-sheets and five α-helices (Hickman et al., 1999). Recently, it was reported that AA-NAT protein activation requires phosphorylation on the Thr^{31} residue and then binding with the chaperone protein 14-3-3 with a ratio 1(AA-NAT)/1(14-3-3 protein). This protein/protein interaction, yielding a relatively stable complex, would lead to conformational changes, unfolding the binding site of the two substrates onto the AA-NAT protein (see Coon et al., 2001; Ganguly et al., 2001, 2002; Obsil et al., 2001; Klein et al., 2002 for reviews). AA-NAT phosphorylation is a crucial step not only because it allows binding to the 14-3-3 protein and activation, but also because it shields AA-NAT from destruction by cytosolic proteasomes (Gastel et al., 1998; Ganguly et al., 2002). Additionally, an intramolecular disulfide bond between the Cys^{-61} and Cys^{177}, formed upon oxidation and cleaved upon reduction, is proposed

to act as a catalytic switch for AA-NAT activation (Tsuboi et al., 2002).

AA-NAT activity is usually measured in saturated concentrations of tryptamine and at optimal pH (6.8) and temperature (37°C) (Deguchi and Axelrod, 1972c; Parfitt et al., 1975). In the rat, the enzyme activity measured during the day is near the detection limit, and between 5 and 20 nmol/h per gland at night. According to our own observations, the mean AA-NAT activity at midday is 0.046 ± 0.015 nmol/gland/h (0.196 ± 0.064 nmol/mg protein/h) and at midnight is 15.06 ± 2.02 nmol/gland/h (62.74 ± 12.13 nmol/mg protein/h). *N*-bromoacetyltryptamine (Khalil et al., 1999) and *N*-chloroacetyltryptamine (Zheng et al., 2001) are potent inhibitors of AA-NAT activity in inducing a reaction of alkyltransferase using another active site of the AA-NAT enzyme.

In the rat pineal gland mRNA expression, protein, and activity of AA-NAT are nearly undetectable during the day and increase markedly (between 70- and 150-fold) during the night (Borjigin et al., 1995; Klein et al., 1996; Roseboom et al., 1996; Gastel et al., 1998; Garidou et al., 2001; Fig. 5). The nocturnal increase in AA-NAT activity requires a neo-transcription of its gene and a neo-synthesis of its protein (Roseboom et al., 1996; Gastel et al., 1998). The protein is very unstable ($t_{1/2}$ about 3 to 5 min), as is the enzyme activity. At the end of the night or following light exposure at night there is a very rapid decrease (within a few minutes) of AA-NAT activity, which is independent of the *Aa-nat* mRNA level and therefore depends mainly upon post-translational mechanisms (Gastel et al., 1998, see below).

Because of the pronounced nocturnal increase in AA-NAT activity observed in the rat pineal gland, this enzyme is usually considered the "rate-limiting enzyme" for the synthesis of MEL. It is noteworthy, however, that there is a high level of NAS release in vitro from NE-stimulated cultured pinealocytes (Miguez et al., 1997) and in vivo in the extracellular medium of microdialyzed nocturnal pineal glands (Azekawa et al., 1991; Sun et al., 2002). These observations suggest that part of the NAS synthesized by AA-NAT is not used by HIOMT to produce MEL, and thus in conditions of marked pineal stimulation HIOMT, rather than AA-NAT, limits MEL synthesis. The predominant feature of AA-NAT in the pineal gland of most species is its large nocturnal increase in activity that drives the daily rhythm in MEL secretion, and as such should be considered the "MEL rhythm-generating enzyme."

Marked differences in the relative importance of the transcriptional, translational, and post-translational mechanisms involved in the nocturnal increase of AA-NAT activity as well as in the amplitude of this increase are observed among species (see Klein et al., 1997, for review; Schomerus et al., 2000; Stehle et al., 2001; see *Section V.B.*).

G. Hydroxyindole-O-Methyltransferase

HIOMT not only catalyzes the final step of the synthesis of MEL, but also that of the other 5-methoxyindoles (Axelrod and Weissbach, 1961; Fig. 4). HIOMT transfers a methyl group from the cofactor *S*-adenosyl-L-methionine to its indolic substrate (Baldessarini and Kopin, 1966). This enzyme represents a large part (2 to 4%) of the pineal proteic fraction (Jackson and Lovenberg, 1971; Sugden et al., 1987b).

The cDNA coding for *Hiomt* was first isolated in the cow (Ishida et al., 1987), then in chicken (Voisin et al., 1992), human (Donohue et al., 1993), rat (Gauer and Craft, 1996), and monkey (Coon et al., 2002), with large species differences noted. The rat cDNA displays low homologies with the cDNA of the cow (65%), human (63%), and chicken (59%). In the rat the whole cDNA sequence is 1728 bp long: the coding region contains 1101 bp, the 5′-noncoding region 184 bp and 3′-noncoding region 444 bp (Gauer and Craft, 1996). The human *Hiomt* gene is the best studied (Donohue et al., 1993; Rodriguez et al., 1994; Bernard et al., 1995). It is located in the pseudoautosomal region of the X chromosome and codes for three transcripts containing a transposable long interspersed element 1 (LINE-1) fragment. Two promoters, containing different *cis*-regulatory elements, have been characterized: one promoter A, whose expression appears restricted to the retina (contains the CCAATTAG sequence able to recognize transcription factors specific for the retina) and one promoter B, containing a CRE and an AP-1 site, whose strong expression in the pineal gland is induced by a pineal specific regulatory element still to be determined (Rodriguez et al., 1994; Fig. 5). This pineal specific regulatory element may be CRX. Indeed, PIRE, the CRX binding site, has been reported in the promoter of human (Li et al., 1998) and chicken (Bernard et al., 2001) *Hiomt*. In addition, CRX binding to *cis*-elements of the chicken *Hiomt* promoter enhances transcription of *Hiomt* (Bernard et al., 2001). The putative amino acid sequence of the rat HIOMT displays 66%, 69%, and 60% homology with that of the cow, chicken, and human, respectively. In the rat, the translated protein is made of 367 amino acids with putative sites of phosphorylation for PKC (3), type II casein kinase (4), and tyrosine kinase (1) (Gauer and Craft, 1996).

In the species studied so far, the enzyme displays a high molecular mass (between 76 and 78 kDa) and is made up of two similar subunits of about 39 kDa each. In the cow, it has been suggested that the subunits could form polymers of a very high molecular weight (Jackson and Lovenberg, 1971). Immunochemical experiments have revealed a large heterogeneity in the protein structure and enzymatic properties among species (Nakane et al., 1983). The protein appears very stable ($t_{1/2} > 24$ h) (Sugden et al., 1987b; Janavs et al., 1991; Bernard et al., 1993, 1995, 1996).

The HIOMT activity assay is performed on pineal homogenates in saturated concentrations of substrate (NAS) and cofactor (*S*-adenosyl-L-methionine), at optimal pH (7.9) and temperature (37°C) according to the method of Axelrod and Weissbach (1960, 1961). The rat pineal HIOMT activity is between 0.7 and 2 nmol/mg protein/h (Sugden et al., 1987b; Ribelayga et al., 1997, 1998b, 1999a,b). HIOMT activity has been measured in the retina and the Harderian gland, although at much lower levels (Quay, 1965; Cardinali and Rosner, 1971; Cardinali and Wurtman, 1972; Nagle et al., 1972, 1973; Pévet et al., 1980a; Wiechmann and Hollyfield, 1989; Bernard et al., 1995; Gauer and Craft, 1996; Djéridane et al., 1998; Ribelayga et al., 1998a). Enzyme studies suggest that the pineal and retina HIOMT are similar, but quite different from HIOMT in the Harderian gland (Cardinali and Wurtman, 1972). These findings are strengthened by the observation that cDNA from the retina can be amplified with specific pineal *Hiomt* primers (Gauer and Craft, 1996). On the contrary, in the Harderian gland, all attempts of RT-PCR amplification and ISH with a pineal *Hiomt* sequence failed (Ribelayga, unpublished observations). Very weak HIOMT activity has also been described in the duodenum and colon, probably in the enterochromaffin cells (Quay and Ma, 1976), in the human retinoblastoma Y79 cell line (Bernard et al., 1995, 1996), and in ovaries (Itoh et al., 1997). RT-PCR experiments have also shown the presence of HIOMT mRNA in human platelets (Champier et al., 1997) and the testis (Poirel and Gauer, personal communication).

In the rat pineal gland, the best substrate for HIOMT is NAS (Axelrod and Weissbach, 1961; Cardinali and Wurtman, 1972; Morton, 1986, 1987). In relative values the enzyme affinity for NAS is between 50 and 80%, for 5-HL between 15 and 30%, for 5-HT around 10%, and for others (5-HTP, 5-HIAA) less than 5%.

In contrast to AA-NAT, the nocturnal increase in pineal HIOMT activity is so low that its occurrence was disputed (for example, see Axelrod et al., 1965 versus Quay, 1967), especially since the activity of the enzyme cannot be stimulated in vivo (Nagle et al., 1973; Ribelayga et al., 1999b) or in vitro (Klein et al., 1970; Berg and Klein, 1971; Ribelayga et al., 1997) by an NAergic agonist. We recently confirmed, however, in several independent studies that rat pineal HIOMT activity displays a weak but significant nocturnal increase (by 40 to 50%) (Ribelayga et al., 1997, 1999b). This increase persists in constant darkness (D/D) and is inhibited in constant light (L/L) (Ribelayga et al., 1999b). Only one study has reported a large (18-fold) nocturnal increase in HIOMT activity of the rat pineal gland (McLeod and Cairncross, 1993).

Hiomt gene expression is already high during the day but still displays a 2-fold increase at night that persists in D/D (Gauer and Craft, 1996; Ribelayga et al., 1999b; Fig. 5). Light exposure at night rapidly ($t_{1/2}$ = 20 min) decreased the level of *Hiomt* mRNA (Ribelayga et al., 1999b). A β-adrenergic receptor (β-AR) agonist stimulated daytime levels of *Hiomt* mRNA, whereas a β-AR antagonist inhibited it (Gauer and Craft, 1996; Ribelayga et al., 1999b). In vitro, neither cAMP nor NAergic agonists stimulated short-term (6 h) HIOMT enzyme activity, suggesting that the nocturnal increase in pineal HIOMT activity does not result from nocturnal stimulation of *Hiomt* gene expression, but rather from NAergic-independent post-transcriptional mechanisms (Ribelayga et al., 1997b, 1999b). However, NPY has been shown to stimulate HIOMT activity (+30 to 40%) in cultured rat pinealocytes within a few hours, suggesting involvement of this peptide in the daily regulation of HIOMT activity (Ribelayga et al., 1997). The short-term regulation of the enzyme appears to involve Ca^{2+} and PKC-dependent mechanisms since its activity can be stimulated by about 30% by thapsigargin or by a phorbol ester (Ribelayga et al., 1997). It is therefore interesting to note that, at least over the short term, the activity and expression of HIOMT appears to be regulated by different neurotransmitters using different mechanisms, suggesting a complex control of this enzyme activity in the rat pineal gland. This hypothesis is strengthened by the ontogenetic study of *Hiomt* gene expression and activity in the rat pineal gland, where the daily variation in *Hiomt* mRNA appeared 10 days before the daily variation in enzyme activity (Ribelayga et al., 1998b).

Several in vivo studies have repeatedly demonstrated that HIOMT activity is regulated over several days/weeks by the nocturnal NAergic stimulation of the pineal gland. Indeed, in the rat, SCGx or exposure to L/L for several days induces a large decrease (2- to 3-fold) of HIOMT activity compared to animals kept in an L/D cycle (Wurtman et al., 1963; Axelrod et al., 1965; Quay, 1967; Moore and Rapport, 1971; Yang and Neff, 1976; Sugden and Klein, 1983b; Ribelayga et al., 1997, 1999b). The decrease in enzyme activity corresponds to a reduction of the quantity of protein (Yang and Neff, 1976). This decrease is abolished by daily injections of an NAergic agonist (Sugden and Klein, 1983a,b,c; Ribelayga et al., 1997). This long-term regulation of HIOMT activity by NAergic stimulation has been confirmed in vitro on long-term cultures of pinealocytes (Ribelayga et al., 1997). The long-term regulation of HIOMT activity is due to high stability of the protein ($t_{1/2}$ > 24h) (Sugden et al., 1987b; Janavs et al., 1991; Bernard et al., 1993, 1995, 1996) and depends upon NAergic control of *Hiomt* gene expression (Ribelayga et al., 1999a, b; see *Section V.A.7.*).

Demonstration of specific regulation of HIOMT activity strongly suggests that this enzyme, in contrast to what is generally described in the literature, is involved in the rhythmic synthesis of MEL, especially the long-term/seasonal rhythm in the nocturnal MEL peak pattern, an important parameter for the transmission of photoperiodic information. During the day AA-NAT ac-

tivity is lower than HIOMT activity and would be the limiting factor for the synthesis of MEL. The increase in AA-NAT activity at the beginning of the night thus induces the increase in MEL synthesis. During the night, however, HIOMT activity is lower than AA-NAT activity and would thus become the limiting enzyme for MEL production. Consequently, any variation in nighttime HIOMT activity should modulate the rate of MEL synthesis (the amplitude of the nocturnal MEL peak). This hypothesis is strengthened by the following observations: 1) an excess of NAS is released in the extracellular compartment at night (Azekawa et al., 1991; Sun et al., 2002) or following NAergic stimulation (Berg and Klein, 1971; Miguez et al., 1997); 2) the NAS concentration can be up to 2-fold greater than that of MEL in the rat (Champney et al., 1984) and the Siberian hamster (Steinlechner et al., 1995); 3) when Siberian hamsters are transferred from LP to SP, the increase in the amplitude of the nocturnal MEL peak is not related to a similar increase in nocturnal AA-NAT activity, but in contrast to a decrease (Illnerova et al., 1984; Ribelayga et al., 2000); and 4) in the Siberian hamster, parallelism between daily variations of NAS and MEL is not always observed throughout along the year (especially in September; Steinlechner et al., 1995).

The above observations have led us to study the long-term, photoperiodic, and seasonal regulation of HIOMT in vitro and in vivo. Using the model of long-term culture of rat pinealocytes, we established an in vitro model with controlled HIOMT and NAT activities. Pinealocytes were cultured for 6 days in the presence or absence of chronic β-adrenergic stimulation (1 μM isoproterenol) then acutely (5 h) stimulated with 1 mM dibutyryl-cAMP in both cases. This gave two conditions: 1) low HIOMT/high AA-NAT and 2) high HIOMT/high AA-NAT. We observed that at equally high AA-NAT activity MEL production is lower when HIOMT activity is lower, strongly indicating that the level of HIOMT activity may limit the amplitude of MEL production (Simonneaux, unpublished data). In vivo, in the rat, we have observed that the duration of the nocturnal peak of *Hiomt* mRNA is longer under SP (8L/16D) than under LP (16L/8D). The SP increase in *Hiomt* gene expression led to a significant increase (30 to 40%) in mean HIOMT activity throughout 24 h, probably related to an augmentation of protein synthesis (Ribelayga et al., 1999a; Fig. 6). In the Siberian hamster pineal HIOMT activity is also twice as high in SP than in LP, while the nocturnal AA-NAT activity, in contrast, is twice as low under SP than under LP. Whatever the photoperiod is, however, both enzymes display similar affinities for their respective substrates, demonstrating that the photoperiodic differences in the enzyme maximal reaction velocity, V_{max}, correspond to differences in enzyme quantity (Ribelayga et al., 2000). In the Siberian hamster, this photoperiodic increase in HIOMT activity parallels a 2-fold increase in the amplitude of the nocturnal MEL peak (Ribelayga et al., 2000). These findings disclose an important physio-

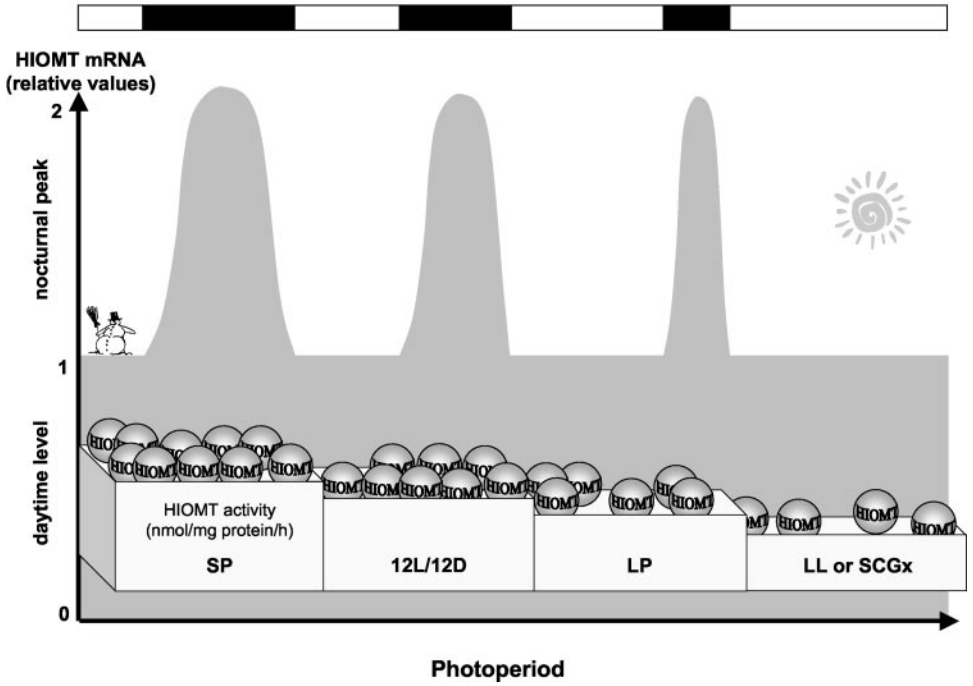

FIG. 6. Photoperiodic regulation of HIOMT activity in the rat pineal gland. The rat *Hiomt* gene is constitutively expressed. In addition, *Hiomt* gene expression is stimulated at night following NAergic stimulation, so that *Hiomt* mRNA levels are increased 2-fold. Under SP (or during winter) the total amount of *Hiomt* mRNA produced daily is higher than under LP (or during summer). Consequently, more HIOMT molecules are produced per day under SP than under LP and due to the high stability of the HIOMT protein, HIOMT activity increases in SP compared to LP. Under the experimental conditions of constant light exposure (L/L) or SCGx, the nocturnal rise in *Hiomt* mRNA is abolished but its basal expression remains. Thus, after a few days in these conditions, HIOMT reaches a low, stable level reflecting basal production of HIOMT molecules.

logical impact of the photoperiodic control of HIOMT activity on seasonal rhythms in MEL secretion (Ribelayga et al., 2000). In the European hamster, HIOMT activity is significantly increased by 80% in November/December in comparison with the earlier months (Ribelayga et al., 1998c). This increase is associated with an increase in the concentrations of 5-ML during the day (Ribelayga et al., 1998c) and MEL at night (Vivien-Roels et al., 1997), suggesting that, in this species as well, HIOMT displays an important role in the photoperiodic control of pineal metabolic activity. We are currently investigating the neurotransmitters and mechanisms implicated in this regulation. In the rat, the photoperiodic variation in HIOMT activity is positively correlated with the rate of *Hiomt* gene transcription, thus suggesting an involvement of the NAergic stimulation (Ribelayga et al., 1999a). In the pineal gland of the European hamster, the increase in HIOMT activity is associated with a large increase in the number of NPYergic fibers but not in TH-positive fibers from the end of October to mid-December (Møller et al., 1998).

The previous observations strongly suggest that HIOMT is involved in the photoperiodic/seasonal modulation of the amplitude of the nocturnal MEL peak observed in several photoperiodic species.

V. Noradrenergic Regulation of Melatonin Synthesis in the Mammalian Pineal Gland

A. Noradrenergic Regulation of Melatonin Synthesis in the Rat Pineal Gland

The observations that the mammalian pineal gland has a dense noradrenergic (NAergic) innervation (Kappers, 1960) and that SCGx suppresses nocturnal MEL synthesis (Moore and Klein, 1974) were the origin of numerous pharmacological, biochemical, and molecular studies designed to delineate the effects of NE on the metabolic activity of the pineal gland. These experiments were performed mainly in the rat, although none of its physiological functions are known to vary according to the photoperiod (see Klein, 1985; Chik and Ho, 1989; Sugden, 1989; Klein et al., 1997 for reviews). Nevertheless, the rat is able to perceive photoperiod changes, to integrate these variations, and to modify the daily synthesis of MEL accordingly. The knowledge acquired in this species model is therefore fundamental. However, it should be kept in mind that marked species differences exist in the nocturnal stimulation of MEL synthesis (see *Section V.B.*). In the rat, NE is the major neurotransmitter involved in the SCN clock control of the metabolic activity of the pineal gland. Rhythmic SCN activity is translated, via positive and negative outputs, as a nighttime stimulation of the SCG fibers (see Buijs, 1996; Moore, 1996 for reviews; Kalsbeek et al., 1999, 2000b). The amount of NE released from the sympathetic fibers is approximately 100-fold higher during the night than during the day (Drijfhout et al., 1996c,d).

The pivotal role of NE in the control of rat pineal metabolic activity has been supported by several experiments: 1) intraperitoneal injections of an NAergic agonist during the day stimulate MEL synthesis with a comparable amplitude to that of the endogenous nocturnal increase (see King and Steinlechner, 1985 for review); 2) SCGx abolishes the nocturnal increase in *Aanat* mRNA, AA-NAT activity, and MEL synthesis (Deguchi and Axelrod, 1972b; Roseboom et al., 1996; Garidou et al., 2001); 3) electrical SCG stimulation during the day provokes an increase in MEL synthesis in the pineal gland (Bowers and Zigmond, 1980); 4) exogenous NAergic stimulation of the pineal gland in organ cultures (Klein and Berg, 1970) or in perifusion (Simonneaux et al., 1989) or of pinealocytes in primary culture (Buda and Klein, 1978; Simonneaux et al., 1994b) induces a large increase in AA-NAT activity and MEL release; 5) the synthetic rate and renewal of NE in the pineal gland are higher at night than during the day (Brownstein and Axelrod, 1974; Craft et al., 1984); and 6) the use of pineal microdialysis to study the in situ regulation of MEL synthesis has demonstrated the positive coupling between the nighttime release of NE and stimulation of MEL synthesis (Drijfhout et al., 1993, 1996c,d).

1. Adrenergic Receptors of the Pineal Gland Several subtypes of adrenergic receptors (AR) are expressed in the rat pineal gland.

a. Subtype β_1 (β_1-AR). This receptor is present at a very high density on the postsynaptic rat pineal membrane (600 fmol/mg protein in the rat pineal gland; Zatz et al., 1976) where it is positively coupled via a G_s protein to the membrane AC (Strada et al., 1972). Its physiological importance has been demonstrated by early in vivo experiments (see Deguchi and Axelrod, 1972a; Romero and Axelrod, 1974; Klein, 1985 for review). The effect of NE appears to be mainly mediated by this receptor subtype since in vivo injections of the β_1-AR agonist isoproterenol (ISO) during the day stimulates AA-NAT activity up to nighttime values (Deguchi and Axelrod, 1972a; Vanecek and Illnerova, 1983) while an in vivo injection of the β_1-AR antagonist propranolol (PROP) strongly inhibits the nocturnal increase in AA-NAT activity (Deguchi and Axelrod, 1972b). The density of the β_1-AR displays a circadian and daily variation, with the highest density observed at the end of the day/beginning of the night (see Romero and Axelrod, 1974; Pangerl et al., 1990, for review). mRNA expression of β_1-AR displays an opposite circadian rhythm, with nighttime values being 2-fold higher than the daytime values (Carter, 1993a; Møller et al., 1997; Pfeffer et al., 1998).

b. Subtype α_1 (α_1-AR). This receptor is localized postsynaptically in the pineal gland (180 fmol/mg protein in the rat; Sugden and Klein, 1984) where it is

coupled to the phospholipase C (PLC) transduction system involving IP_3, Ca^{2+}, and DAG (Klein, 1985). The mRNA coding for both types 1A and 1B is expressed in the rat pinealocytes, but only the protein of the 1B subtype appears to be present (Sugden and Klein, 1984; Sugden et al., 1996). The mRNA expression of these receptors displays a circadian and daily variation, with higher values at night (Coon et al., 1997). The receptor density, however, shows no daily variation, but increases 2-fold after 3 weeks in L/L or after SCGx (Sugden and Klein, 1985) suggesting a slow turnover of the receptor protein.

c. Subtype α_2 (α_2-AR). This receptor has been characterized pharmacologically as the α_2-ARA/D subtype (70 fmol/mg protein in the bovine and rat pineal glands, Simonneaux et al., 1991a; Schaad and Klein, 1992). Several in vivo and in vitro studies have shown that this receptor is localized on the presynaptic NAergic terminals, where it inhibits NE release (Pelayo et al., 1977; Simonneaux et al., 1994b). Other studies, however, have shown that this receptor is also present on the pinealocyte membranes, where it indirectly activates a guanylate cyclase (GC) (Venkataraman et al., 1998) and stimulates AA-NAT activity (Schaad and Klein, 1992) and MEL release (Mustanoja et al., 1999).

2. Second Messengers Induced by Noradrenergic Stimulation. NAergic stimulation of the rat pineal gland at night induces various intracellular events (Fig. 5):

1. Intracellular cAMP levels are increased about 100 times (Strada et al., 1972). This action of NE is initiated by the β_1-AR positively coupled by a G_s to AC, which expression is maximal at night (Tzavara et al., 1996). The activation of these β_1-AR only leads to a 10-fold increase in the cAMP level, the maximal increase in cAMP levels is actually reached when the α_1-AR are activated at the same time (Vanecek et al., 1985). Activation of the α_1-AR alone has no effect on cAMP, but it does potentiate the β_1-AR-induced increase in cAMP levels probably via a type I PKCa^{2+}/CaM (Tzavara et al., 1996). The main effect of the cAMP increase is to stimulate pineal PKA activity (Fontana and Lovenberg, 1971). A clear role for type II PKA in the cAMP-mediated control of MEL synthesis has been demonstrated, although a participation of type I PKA is not excluded (Maronde et al., 1999b).
2. Intracellular cGMP concentration is also increased about 100 times following NAergic stimulation (Vanecek et al., 1985). As for regulation of cAMP, the activation of the β_1-AR alone leads to a 2- to 5-fold increase in cGMP levels (Sugden, 1990b) while activation of both β_1-AR and α_1-AR induces a 20-fold further increase (Chik and Ho, 1989). It appears that the β_1-AR-induced increase in cGMP is mediated by a G_s-protein-coupled GC, while α_1-AR potentiation involves activation of nitric oxide (NO) synthetase (NOS) and the production of NO, which stimulates cytosolic GC, and therefore cGMP production (Spessert et al., 1993; White and Klein, 1993, 1995). This finding is in agreement with the following observations: Na^+ nitroprusside (NO donor) stimulates cGMP accumulation in rat pinealocytes (White and Klein, 1993); NE-induced cGMP accumulation is inhibited by NOS inhibitors (Lin et al., 1994); and the Ca^{2+}/CaM sensitive form of NOS is present and stimulated by NE (Lin et al., 1994). Na^+ nitroprusside alone is able to stimulate cGMP accumulation, indicating that the role of β_1-AR activation would be to make the NOS responsive to the Ca^{2+}/CaM complex. The β_1-AR are also involved in the long-term regulation of NOS activity (Schaad et al., 1994, 1995a). cAMP analogs have no effect on cGMP levels whether they are used alone or with NO donors (White and Klein, 1995).
3. Intracellular levels of Ca^{2+} (Ca^{2+}_i) are increased following NAergic stimulation (Sugden et al., 1987a; Saez et al., 1994; Schaad et al., 1995b; Schomerus et al., 1995; Marin et al., 1996; Simonneaux et al., 1999). This cellular event results from the specific activation of the α_1-AR that are coupled to the PLC transduction system and Ca^{2+} channels (Chik and Ho, 1989). The Ca^{2+}_i increase is biphasic with an initial rapid and transient peak resulting from Ca^{2+} release from the IP_3-sensitive intracellular stores, and a second sustained response resulting from the opening of membrane Ca^{2+} channels. This increase in Ca^{2+}_i induces the translocation and activation of PKC (Ho et al., 1988a). It is noteworthy that in the pineal gland, PKC is mainly activated by Ca^{2+} and much less (about 10%) by DAG (Ho et al., 1988b). It appears, therefore, that the stimulatory effect of α_1-AR on cyclic nucleotide accumulation is mainly mediated by Ca^{2+} and PKC. However, cAMP potentiation requires PKC activation or a Ca^{2+} increase, whereas cGMP potentiation requires both PKC activation and Ca^{2+} increase (Sugden et al., 1985b; Ho et al., 1987a; Spessert et al., 1995). It is suggested, therefore, that the α_1-AR potentiation of cAMP accumulation induced by Ca^{2+} occurs via PKC acting on G_s or AC (Sugden and Klein, 1988), whereas α_1-AR potentiation of cGMP is mediated by a Ca^{2+}/PKC complex and a PKCa^{2+}/CaM since it also requires activation of a PKCa^{2+}/CaM-dependent NOS (Ho et al., 1991; White and Klein, 1995). Several isoforms of PKC are present in the rat pineal gland, with different effects (Ogiwara et al., 1998): the specific inhibition of α and β(1) PKC isoforms by Go6976 ($C_{24}H_{18}N_4O$) leads to a surprising increase in NE-stimulated cAMP and cGMP levels, whereas the nonspecific PKC inhibitor cal-

phostine C reduces the effect of NE. These data suggest that some PKC (those sensitive to Go6976) exert a tonic inhibition on cyclic nucleotide levels (maybe through a phosphodiesterase), while others potentiate β_1-AR stimulation of cyclic nucleotide synthesis.

4. Phospholipase A_2 activity and subsequent arachidonic acid synthesis are increased following activation of α_1-AR (probably through a PKC-dependent mechanism; Ho and Klein, 1987). Arachidonic acid metabolites may be involved in cGMP formation (Chik et al., 1991).
5. Pinealocyte membrane hyperpolarizes following NAergic stimulation (Parfitt et al., 1975). This effect results, at least partly, from a K^+ efflux provoked by the opening of a Ca^{2+}-sensitive K^+ channel (Cena et al., 1991). Activation of this channel requires an increase in both cAMP and Ca^{2+} levels (Cena et al., 1991). The intracellular pH measured in nonstimulated rat pinealocytes is 7.09 and increases up to 7.20 following 10 μM NE stimulation (Ho et al., 1989). This cytosol alkalinization results from the α_1-AR activation that produces the opening of an Na^+/H^+ antiport (Ho et al., 1989). This alkalinization is thought to facilitate NE stimulation of cyclic nucleotide content (Vanecek et al., 1986; Ho et al., 1992). Some clusters of cultured pinealocytes display action potentials whose frequency is modulated by β_1-AR activation (Schenda and Vollrath, 1998). However, it is questioned whether these endogenous action potentials are a phylogenetic remnant of the lower vertebrates' pineal clock.
6. Phosphorylation of mitogen-activated protein kinase (MAPK). In rat pinealocytes, the presence of p42 and p44 isoforms of MAPK has been established (Kiyama et al., 1994; Ho et al., 1999). In addition, the presence of two upstream elements involved in the regulation of MAPK, namely MEK1 and Raf1, has been reported (Ho et al., 1999). NE alters MAPK phosphorylation through a dual mechanism: stimulation of the cAMP/PKA pathway inhibits while activation of the cGMP/PKG transduction cascade stimulates MAPK phosphorylation (Ho and Chik, 2000). However, the overall effect of NE on MAPK phosphorylation is stimulatory via the cGMP/PKG pathway (Ho et al., 1999). To date, the function of MAPK in rat pinealocytes is still not known and its effect on MEL synthesis has not been investigated.

3. The Third Messengers/Transcription Factors Induced by Noradrenergic Stimulation. The NE-induced increase in second messengers leads to activation of several transcription pathways: 1) phosphorylation of the transcription factor CREB, which then activates transcription of genes coding for the MEL-synthesizing enzymes; 2) expression of the mRNA coding for the immediate early genes (IEG); 3) expression of clock genes; and 4) expression of specific pineal and retinal transcription factors. In addition, the cDNA array analysis of pineal gene expression may help to discover additional genes coding for transcriptional regulators as, for example, the rat pineal Id-1 gene encoding a helix-loop-helix protein (Humphries et al., 2002).

1. The transregulator element CREB is constitutively present in the pineal gland. Stimulation of β_1-AR, but not α_1-AR, induces a large and rapid phosphorylation of CREB into P-CREB (Roseboom and Klein, 1995) in nearly all pinealocytes (Tamotsu et al., 1995). CREB is usually phosphorylated by PKA. Although Ca^{2+} ionophores, α_1-AR agonists, or cGMP analogs have no effect on CREB, application of ouabain or a high KCl concentration (which depolarizes the cells) results in CREB phosphorylation (Roseboom and Klein, 1995). This latter effect could be induced by the type I or IV PKCCa^{2+}/CaM, which is able to phosphorylate CREB on the Ser^{133} and induce its activation (Sun et al., 1996). In the rat pineal gland, however, CREB phosphorylation is mainly induced by the β_1-AR/AC/cAMP/PKA transduction pathway (Roseboom and Klein, 1995). P-CREB is a key element in the regulation of pineal gene expression, therefore regulatory mechanisms involved in P-CREB dephosphorylation deserve careful study.

P-CREB enhances expression of the genes coding for the enzymes of MEL synthesis, which are endowed with putative CRE sites in their promoter region reported as *Aa-nat* (Baler et al., 1997; Burke et al., 1999) and *Hiomt* (Rodriguez et al., 1994). In addition, stimulation of *Aa-nat* (Roseboom et al., 1996) and *Hiomt* (Ribelayga et al., 1999b) gene expression is not inhibited in the presence of the protein synthesis inhibitor cycloheximide, suggesting that a constitutive element such as CREB is involved (after phosphorylation) in the nocturnal stimulation of the expression of these genes (see Foulkes et al., 1996a, 1997 for reviews). It has been recently stressed, however, that P-CREB does not totally account for the entire cAMP effect on *Aa-nat* gene expression; the phosphatase inhibitor okadaic acid, which increases P-CREB independently of cAMP formation, does not induces *Aa-nat* gene expression, and induces low *Icer* gene expression but full *Fra2* and *JunB* mRNA (Spessert et al., 2000); cAMP may activate AP-1-binding activity besides CREB phosphorylation (Carter, 1994); and activation of both the CRE and the CCAAT sites of the *Aa-nat* promoter are needed for full *Aa-nat* gene expression (Baler et al., 1997).

In addition, P-CREB induces expression of a CRE-related gene that modulates the cAMP response, CRE modulator (*Crem*) (Stehle et al., 1993). The *Crem* gene is transcribed into different splice variants that are translated into proteins that activate (CREM α, β, γ) or inhibit (CREM τ) CRE activity according to the tissue or developmental state (see Foulkes and Sassone-Corsi,

1996 for review). Consequently, the protein CREM will act either in synergy with P-CREB or compete with P-CREB on the CRE sites. In the pineal gland, *Crem* is strongly expressed in the form of a short size transcript. In contrast to the other members of this family, its expression is inducible by cAMP. It was found to code for a protein exerting a strong inhibitory effect on cAMP-induced transcription and was named after this effect, inducible cAMP early repressor (ICER) (see Stehle et al., 1993, 2001 for review). A *cis*-regulatory element that binds CREB, but with a sequence slightly different from that of CRE, is present in four copies in the promoter region of *icer* and is named CARE (for CRE-like element: TGATGTCA) (Foulkes and Sassone-Corsi, 1996). There is a marked circadian rhythm of *Icer* expression with higher levels at night (approximately 100-fold) compared to daytime. *Icer* expression is induced by the cAMP-dependent pathway triggered by β_1-AR activation and peptides like VIP or PACAP (Stehle et al., 1993; Foulkes and Sassone-Corsi, 1996; Pfeffer et al., 1999). In the perifused pineal gland of intact rats, *Icer* expression is induced by ISO or cAMP analogs when applied at the end of the day/beginning of the night, but not during the day, supposedly because of the presence of the inhibitory transcription factor ICER, this system representing a negative feedback loop. This inhibitory effect of ICER could be extended to all the other CRE sites, especially that of the *Aa-nat* gene. This finding led Stehle et al. (1993) to consider ICER as an element responsible for the decrease in MEL synthesis at the end of the night. However, this attractive hypothesis appeared not valid when the decrease in AA-NAT activity and MEL synthesis was found to occur a few hours before the decrease of *Aa-nat* gene expression (Roseboom et al., 1996; Ribelayga et al., 1999a). There is a daily variation in pineal ICER immunoreactivity, although with a relatively smaller nighttime increase (approximately 4-fold) occurring toward the late night/early day (ZT 18–0); in contrast, the stimulatory P-CREB peaks earlier at night (ZT 16–20) and declines toward the end of the night (ZT 22), suggesting that the inhibitory transcription factor inhibits the cAMP-stimulated genes, particularly *Aa-nat*, toward the end of the night (Maronde et al., 1999a). This is strengthened by the finding that *Aa-nat* gene expression is increased following the *Icer* gene silencing either in vivo (Foulkes et al., 1996a) or in vitro (Maronde et al., 1999a; Pfeffer et al., 2000). It is possible that any pineal gene whose promoter contains a CRE site may have its expression down-regulated by ICER as reported for the β_1-AR coding gene (Pfeffer et al., 1998). In addition, it has also been reported that *Icer* gene expression displays photoperiodic variation, suggesting that the quite stable ICER protein may be involved in the long-term (photoperiodic) regulation of the cAMP-inducible expression of genes, and therefore MEL synthesis (Foulkes et al., 1996b; see *Section V.A.7.*).

2. Several IEGs are expressed in the rat pineal gland following NAergic stimulation. The corresponding proteins of the IEGs form homo- or hetero-dimers to become AP-1 transcription factors. The expression of *c-fos*, *c-jun*, *junB*, *junD*, *NGFI-A*, and *Fra-2* has been characterized in the rat pineal gland (see Baler and Klein, 1995; Carter, 1997 for review). IEG expression can be initiated by PKA (via P-CREB/CRE) or PKC. Translation of *IEG* mRNA into protein is very rapid (30–60 min). NAergic stimulation alters the expression of some pineal *IEG*s. The relative role of both AR-types and associated transduction pathways differs according to *IEG*. Expression of *c-fos* mRNA displays a transient and rapid increase at the beginning of the night, and then decreases gradually during the night (Carter, 1990; Koistinaho and Yang, 1990, 1992). This increase is probably mediated by α_1-AR (Carter, 1992, 1993b), although ISO or dibutyryl-cAMP may also induce FOS expression in cultured rat pinealocytes (Tuulivaara and Koistinaho, 1991). Expression of *junB* follows a pattern similar to that of *c-fos* (Carter, 1992), but the nocturnal increase is under the dependence of both β_1-AR and α_1-AR activation (Carter, 1992, 1993c). The level of *junD* expression does not vary in the course of the day and the application of AR agonists or antagonists has no effect on its expression (Carter, 1992). The expression of *c-jun* is partially suppressed during the night following β_1-AR activation (Carter, 1992). However, in vitro expression of *c-jun* is stimulated by NE. This activation would result from the antagonistic effects of two transduction pathways one excitatory (PKC) and one inhibitory (PKA) (Carter, 1992, 1993b). The regulation of *Fra-2* expression has been particularly well characterized (Baler and Klein, 1995). The *Fra-2* mRNA and protein levels are undetectable during the day and increase markedly at night. These variations are circadian and depend mainly on β_1-AR regulation of the cAMP levels. The increase in $Ca^{2+}{}_i$ or cGMP, or the α_1-AR activation, has no effect on *Fra-2* expression (Baler and Klein, 1995). The expression of *NGFI-A* increases at the beginning of the night, then remains elevated throughout the night, probably as a result of both β_1-AR and α_1-AR coactivation (Carter, 1992).

The temporal distribution of the expression of the various *IEG*s results in quantitative and qualitative variations in the composition of the heterodimers in the course of the daily cycle. Interestingly, junB and Fra-2 appear as major nocturnal players since 1) they accumulate in the pineal gland during the nocturnal phase; 2) their repressor effect on transcriptional activity has been established in many tissues; and 3) AP-1 activity in the pineal gland displays a daily variation with higher values during the nocturnal phase that mainly results from the effect of Fra2 and junB (Carter, 1994, 1997; Klein et al., 1997; Guillaumond et al., 2000). Fra2 was expected to be an inhibitory transcription factor involved in the decrease in *Aa-nat* mRNA in the morning

(Klein et al., 1997). However, this hypothesis is ruled out by the recent finding that pineal *Aa-nat* gene expression is not altered in transgenic rats with a dominant negative *Fra2* gene (Smith et al., 2001). The daily AP-1 variation in the pineal gland is probably involved in some other transcriptional regulation. To date, however, no functional relationship has been established between the induction of *IEG*s and daily changes in MEL synthesis (see Baler and Klein, 1995; Carter, 1997 for reviews).

3. Expression of clock genes has been reported recently in the mammalian pineal gland. Since recent data have demonstrated that numerous tissues, besides the SCN, are endowed with the molecular clock machinery (Balsalobre et al., 1998; Yamasaki et al., 2000), it was logical to look for the expression of clock genes in the mammalian pineal gland. *Bma1l, Clock* (Namihira et al., 1999), *Per1* and *Per2* (Fukuhara et al., 2000; Takekida et al., 2000; von Gall et al., 2001), *Per3* (Simonneaux, unpublished data), and *Cry1* and *Cry2* (Nakamura et al., 2001; Simonneaux, unpublished data) are all expressed in the rat pineal gland.

Per1, *Per2*, *Per3*, *Cry1*, and *Cry2* mRNA display daily variations with a nocturnal increase peaking 2 h before that of *Aa-nat* mRNA (Fukuhara et al., 2000; Takekida et al., 2000; Simonneaux, unpublished data). The nocturnal increases of *Per1* mRNA and PER protein (Takekida et al., 2000; von Gall et al., 2001; Fukuhara et al., 2002) and of *Cry2* mRNA (Simonneaux, unpublished data) are induced by the NE/β_1-AR/cAMP pathway. Surprisingly, the daily variations in *Per2* (Takekida et al., 2000; Fukuhara et al., 2002), *Per3* and *Cry1* (Simonneaux, unpublished data) expression do not appear regulated by β_1-AR ligands. *Clock* and *Bmal1* expression displays slightly opposite daily variations, with *Bmal1* mRNA being a little higher during the day (Namihira et al., 1999).

The role of the circadian clock components in the mammalian pineal gland is intriguing and still needs to be delineated. Transfection experiments in rat pinealocytes revealed, in contrast to what was observed in retinal photoreceptors, a surprising inability of CLOCK/BMAL1 to induce E-box-mediated stimulation of *Aa-nat* gene expression (Chen and Baler, 2000). It was recently reported that *mPer1*-luciferase activity oscillates for two to three circadian cycles in isolated rat pineal glands (Abe et al., 2002) and that *Per1* expression may be stimulated by CLOCK/BMAL1 in transfected pinealocytes (Fukuhara et al., 2002). Experiments are now required to delineate whether the pineal clock proteins are only a reminiscence of the lower vertebrate clock pineal or display specific functions in the mammalian pineal gland (investigating the effect of clock gene silencing on MEL synthesis and photoperiodic regulation).

4. A specific transcription factor has been characterized in the pineal gland and the retina (Li et al., 1998). The pineal gland and retina contain the specific transcription factor CRX that may regulate their differentiation and drive the spatial expression of genes exclusively expressed in the photoreceptors and pinealocytes. CRX binds the *cis*-regulator PIRE site (TAATC/T), which is found in the promoter of *Aa-nat* (3 copies) and *Hiomt* (1 copy in each of the A and B promoters). The *Crx* gene is highly expressed in the pineal gland and displays a 3-fold nocturnal increase with a peak preceding that of *Aa-nat* mRNA. Recently, the importance of CRX in MEL synthesis was highlighted by the report that *Aa-nat* gene expression is strongly reduced in *Crx*-deficient mice (Furukawa et al., 1999). These observations suggest that CRX could play an important function in the regulation of pineal gene expression and may be in synergy with the β_1-AR/cAMP/PKA/P-CREB pathway.

4. Acute Effects of Noradrenergic Stimulation on the Melatonin Synthesis Pathway. 1) Activation of the cAMP/PKA pathway is the major nocturnal event that stimulates MEL synthesis (Klein and Berg, 1970; Klein et al., 1970, 1996; Berg and Klein, 1971; Roseboom et al., 1996). Although the daytime levels of *Tpoh* mRNA and activity are elevated, activation of the cAMP/PKA pathway induces a small (13%) nocturnal increase in *Tpoh* gene expression, probably through the effect of P-CREB (Besançon et al., 1996, 1997) and phosphorylation/activation of TPOH (Johansen et al., 1995, 1996). These events result in a 2-fold nocturnal increase in TPOH activity (Ehret et al., 1991). Importantly, the cAMP-induced activation of PKA has several effects on AA-NAT activation. First, the nocturnal increase in cAMP-dependent P-CREB induces a massive (100–150-fold in the rat) nocturnal increase in *Aa-nat* gene expression (Borjigin et al., 1995; Roseboom et al., 1996; Garidou et al., 2001). Translation of *Aa-nat* mRNA results in a large increase (70–100-fold) in the protein level. Second, PKA phosphorylates the Thr^{31} residue of AA-NAT, which in turn binds to the 14-3-3 chaperone protein to become an activated enzyme (Ganguly et al., 2001, 2002). Third, phosphorylated AA-NAT is protected from proteasome proteolysis (Gastel et al., 1998; Ganguly et al., 2001). Nocturnal AA-NAT activation therefore requires transcriptional, translational, and post-translational mechanisms mainly triggered by the cAMP/PKA pathway (see Ganguly et al., 2002 for review). The phosphorylated AA-NAT/14-3-3 complex binds serotonin and acetylCoA with high affinity and converts serotonin into *N*-acetylserotonin. The daytime level of *Hiomt* mRNA is rather high but still increases further (2-fold) following nocturnal activation of the cAMP/PKA pathway (Gauer and Craft, 1996; Ribelayga et al., 1999b). This effect does not require de novo protein synthesis and is therefore induced by a constitutive protein (Ribelayga et al., 1999b), which may be P-CREB as indicated by the presence of a CRE site in the gene promoter. The activity of HIOMT, however, is not acutely stimulated by cAMP analogs, ISO or NE (Klein et al., 1970; Berg and Klein, 1971; Ribelayga et al., 1997, 1999b). The marked nocturnal increase in AA-NAT activity following activation

of the β_1/AR/cAMP/PKA pathway induces a large conversion of 5-HT (its levels therefore decreasing at night) into NAS, then MEL. 2) A nocturnal increase in $Ca^{2+}{}_i$ and activation of PKC modulate MEL synthesis. The NE-induced $Ca^{2+}{}_i$ increase potentiates the intracellular elevation of cAMP levels and consequently AA-NAT activation and MEL synthesis. This potentiating effect results mainly from PKC action on AC activity (Sugden et al., 1985b). In addition, a specific α_1-AR agonist alone increases AA-NAT mRNA and activity to a small extent (Roseboom et al., 1996), indicating Ca^{2+}-dependent stimulation of *Aa-nat* gene transcription (may be via an AP-1 site) and protein activation (may be via a PKC phosphorylation site). In vitro, drugs like ionomycin and calcimycin (A23187), which artificially raise $Ca^{2+}{}_i$, increase AA-NAT activity without elevation of cAMP and P-CREB levels. This suggests the involvement of another pathway in the transduction of this effect or a direct effect of Ca^{2+} on the enzyme (Yu et al., 1993). In contrast, a high concentration of KCl or ouabain induces CREB phosphorylation (Roseboom and Klein, 1995), but blocks the AA-NAT response to cAMP (Parfitt et al., 1975). It is possible that depending upon the mechanisms by which Ca^{2+} is mobilized in the cell, activation of transduction systems could be specific and distinct. Similar multiple mechanisms in Ca^{2+} regulation have been observed in neurons of the hippocampus (Bading et al., 1993).

No direct effect of cGMP analogs on the activity of the MEL-synthesizing enzymes has been observed on either AA-NAT (Seidel et al., 1990) or HIOMT activity (Ribelayga, unpublished observations). Consequently, cGMP has no effect on the synthesis and release of MEL (Spessert et al., 1992; Lin et al., 1994). However, some effects of cGMP on pineal biochemistry have been reported. cGMP inhibits an L-type Ca^{2+} channel, probably through the activation of PKG (Chik et al., 1995). Closing of this channel following cell hyperpolarization and cGMP accumulation induced by NE is thought to participate in membrane stability (Chik et al., 1995). The pineal gland also displays a cGMP-sensitive cationic channel similar to that of rod photoreceptors (Schaad et al., 1995b). Activation of this channel by cGMP increases $Ca^{2+}{}_i$ (Schaad et al., 1995b). More generally, cGMP is thought to be involved in $Ca^{2+}{}_i$ homeostasis (see Milbourne and Bygrave, 1995 for review) by activating Ca^{2+}-dependent ATPase of the endoplasmic reticulum and inhibiting the IP_3 receptor through PKG-dependent phosphorylation. Recently, cGMP was reported to induce *Per1* gene expression via the MAPK pathway (Fukuhara et al., 2002).

5. Mechanisms Involved in the Termination of Nocturnal Melatonin Synthesis. In the rat, irrespective of the photoperiod, the synthesis of MEL starts to decrease before the end of the dark phase (Tamarkin et al., 1985; Ribelayga et al., 1999a). This diminution results from various cellular and molecular mechanisms mainly initiated by the termination of NE release (Drijfhout et al., 1996d). Cessation of NE release is thought to be SCN clock-driven but also depends on local presynaptic inhibition via α_2-AR (Pelayo et al., 1977; Simonneaux et al., 1994a). Termination of NAergic stimulation results in a rapid decrease in the intracellular levels of cAMP (Klein et al., 1978) and consequently to:

1. A large, rapid decrease in AA-NAT activity resulting from termination of its cAMP/PKA-dependent protection (Fig. 5). With the decrease in cAMP levels and PKA activity (Winters et al., 1977), AA-NAT is dephosphorylated, released from the 14-3-3 protein, and then subjected to a rapid proteolysis by the cytosolic proteasome (Gastel et al., 1998; Ganguly et al., 2001, 2002). The decrease in AA-NAT activity is immediately followed by a decline in MEL synthesis and release.
2. Cessation of the nocturnal stimulation of gene expression coding for *Tpoh, Aa-nat,* and *Hiomt.* These events, however, are without immediate effect on the synthesis of MEL since reduction of *Aa-nat* and *Hiomt* mRNA occurs after the decrease in MEL levels (Roseboom et al., 1996; Ribelayga et al., 1999a). The half-life of the mRNA is approximately 2.5 h for *Aa-nat* (Roseboom et al., 1996) and less than 2 h for *Hiomt* (Ribelayga et al., 1999b). A decrease in the expression of these genes at the end of the night/beginning of the day may simply result from termination of NAergic stimulation and a consecutive P-CREB dephosphorylation and/or from accumulation of the inhibitory transcription factor ICER (Maronde et al., 1999a). However, it should be noted that in *Crem*-deficient mice *Aa-nat* mRNA levels display a higher amplitude but decrease at the same time in the late night (Foulkes et al., 1996a).
3. Other cellular mechanisms: β_1-AR are desensitized toward the end of the night (Pangerl et al., 1990; Freedman et al., 1995); a feedback effect of PKC on the α_1-AR-induced increase in $Ca^{2+}{}_i$ occurs (Sugden et al., 1988); specific phosphatases may inhibit NE-induced cyclic nucleotide production and CREB phosphorylation (Ho and Chik, 1995); the size of the *Aa-nat* transcript decreases at night, reflecting a reduction in the polyadenylated tail, a mechanism known to decrease transcript stability and translation efficiency (Roseboom et al., 1996); a decrease in AA-NAT activity could also result from a mechanism of protein thiol:disulfide interaction (Namboodiri et al., 1981); *S*-adenosyl-L-homocysteine, which accumulates during the night, may inhibit HIOMT activity (Tedesco et al., 1994); and finally, reduced MEL synthesis could result from a decrease in the quantity of its substrates.

In the rat pineal gland, proteolytic degradation of AA-NAT resulting from termination of NE-induced stimulation of cAMP appears as the main event respon-

sible for ending MEL synthesis toward the end of the night (Gastel et al., 1998). A similar mechanism has been reported in other species (Klein et al., 1997; Schomerus et al., 2000), thereby suggesting that this mechanism is a common one shared across species.

6. Effect of Light Exposure at Night. Acute light exposure at night induces a rapid and complete inhibition of AA-NAT activity and MEL synthesis in the rat pineal gland (Klein and Weller, 1972; Illnerova et al., 1979). A 1-min light pulse is sufficient to reduce AA-NAT activity and MEL concentrations to daytime values within 20 min (Vanecek and Illnerova, 1979; Drijfhout et al., 1996c). Inhibition by light can be produced by light intensity as weak as 0.5 lux (Vanecek and Illnerova, 1982).

This rapid inhibitory effect of light seems rather complex, as it appears to involve various sequential events and several neural structures and pathways. Previous electrophysiological studies have shown that light exposure at night induces an evoked response in the pineal gland (Dafny, 1980). By applying a local anesthetic in the SCG or performing SCGx, this author has shown that the light-induced response is composed of two components: a rapid component going through a central nervous pathway and a slower component transmitted via the SCG (Dafny, 1980). The rapid component of light-induced inhibition of AA-NAT activity could perhaps follow a central pathway originating in the retina and going through the IGL (a structure known to display FOS reactivity in response to light exposure at night in the rat; Peters et al., 1996) and deep pineal (Cipolla-Neto et al., 1995; Bartol et al., 1997). IGL fibers contain NPY and GABA, both of which have been shown to inhibit NE release in vitro (GABA: Rosenstein et al., 1990; NPY: Simonneaux et al., 1994b). However, the transmitter(s) and mechanism(s) involved in this effect remain hypothetical. The other component of light inhibition arises from the SCN, which drives a slower, more sustained inhibition of NE release via the SCG sympathetic fibers. Even though postsynaptic inhibitory mechanisms exist for AA-NAT activity, it is more probable that light-induced inhibition of MEL synthesis essentially results from the very rapid termination of NE release ($t_{1/2} < 10$ min) (Drijfhout et al., 1996c). Cessation of NAergic stimulation induces a rapid decrease in the intracellular concentration of cAMP and a consequent fast ($t_{1/2} < 2$ min) degradation of AA-NAT protein by proteasome, independent of the *Aa-nat* mRNA level (Gastel et al., 1998).

It is known that light exposure at night differentially affects the circadian clock machinery depending upon whether it is applied in the first or the second part of the night (Reppert and Weaver, 2001). Similarly, in the pineal gland, when a 1-min light pulse is applied during the first part of the night (before ZT 18 in rats kept in 12:12 L/D) AA-NAT activity and MEL synthesis decrease but increase again the same night. However, if the light pulse is applied during the second part of the night (after ZT 19) AA-NAT activity and MEL synthesis remain low for the rest of the night (Illnerova and Vanecek, 1985). We propose that the latter observation, applying light after ZT 19, results from a clock-dependent inhibition of NE release since it is possible to reinduce *Aa-nat* mRNA and MEL release by injection of a β_1-AR agonist during or 1 h after the late light pulse (Saboureau, Garidou, and Simonneaux, unpublished data) independently of the presence of ICER (Maronde et al., 1999a).

7. Consequences of Long-Term Noradrenergic Stimulation of the Pineal Gland. The long-term (few weeks) consequences of repeated nocturnal NAergic stimulation of the pineal gland are observed on proteins with long half-lives (over 24 h). This was studied in experimental conditions that produced total suppression of pineal NAergic stimulation (by SCGx, decentralization of the SCG, or keeping animals in L/L) or modulated the duration of NAergic stimulation (raising animals in different photoperiods).

1. Pineal HIOMT activity is regulated in the long term (Axelrod et al., 1965; Sugden and Klein, 1983a,b; Ribelayga et al., 1997; Fig. 6) but this regulation depends on repeated nocturnal stimulation of *Hiomt* gene expression (Ribelayga et al., 1999b). The nocturnal NAergic stimulation of *Hiomt* gene expression, although having no direct effect on the nocturnal increase of HIOMT enzyme activity, is required for the synthesis of supplementary enzyme and to maintain constant basal HIOMT activity. In the absence of NAergic stimulation (for example, when animals are SCGx or raised under L/L), the nocturnal peak of *Hiomt* gene expression disappears but the daytime level of *Hiomt* mRNA is maintained throughout the 24 h period for up to 2 weeks (Ribelayga et al., 1999b). In these conditions, HIOMT activity slowly decreases down to about 50% of its initial value within two weeks and stabilizes at this level, not decreasing any further (Sugden and Klein, 1983a,b). This basal value probably results from daytime synthesis *Hiomt* gene expression (Ribelayga et al., 1999b). When animals are exposed to an L/D cycle, the release of NE induces a nocturnal peak of *Hiomt* mRNA. The increased amount of mRNA over 24 h gives a higher amount of protein, and finally the balance between protein synthesis and degradation stabilizes at the basal HIOMT activity observed in L/D cycle (Ribelayga et al., 1999b). To test this hypothesis, we have studied mRNA expression and enzyme activity of HIOMT in rats raised in different photoperiods. In accordance with our hypothesis, we have observed that an increase in the duration of the night results in an increase in the duration of the nocturnal peak of *Hiomt* mRNA and in the mean daily HIOMT activity (Ribelayga et al., 1999a). Interestingly, when the length of the dark phase is over 12 h, the duration of the *Hiomt* mRNA peak no longer increases, nor does the mean HIOMT activity. This confirms the correlation between quantity of nocturnal mRNA and

mean level of HIOMT activity. These observations suggest that the photoperiodic regulation of HIOMT activity directly depends on NE-induced transcriptional mechanisms (Fig. 6). Similar photoperiodic regulation of HIOMT activity has also been suggested in European (Ribelayga et al., 1998c) and Siberian (Ribelayga et al., 2000) hamsters.

2. A role for ICER in the pineal gland was demonstrated in long-term experiments (Foulkes et al., 1996b). Duration of the nocturnal peak of *Icer* gene expression, and consequently the level of ICER protein, is proportional to the duration of the night with higher ICER levels in the pineal of rats raised in SP compared to LP. In SP, the presence of high levels of ICER at the beginning of the night results in a reduction of P-CREB activity (Foulkes et al., 1996b) and thus in cAMP-dependent mRNA expression. In contrast, in LP, the lower levels of ICER at the beginning of the night favor more rapid induction of these mechanisms than in SP. In the pineal gland, therefore, the photoperiodic variation in ICER level (with higher levels in SP) may control the photoperiodic variation in the pattern (slope of the increase and amplitude) of the nocturnal expression of the MEL-synthesizing enzymes. In support of this hypothesis, we have observed that the amplitude of the nocturnal peak of pineal *Aa-nat* mRNA and activity is lower in SP than in LP in rodents: rat (Ribelayga et al., 1999a), Siberian hamster (Ribelayga et al., 2000), and Syrian hamster (Garidou et al., 2003a). The role of ICER in the modulation of genetic expression was demonstrated in the pineal gland of *Crem*-deficient transgenic mice, in which the amplitude of the *Aa-nat* mRNA nocturnal peak was markedly increased (Foulkes et al., 1996a). The ICER protein may modulate the rate and magnitude of MEL induction throughout the 24 h cycle. By binding CRE in the *Aa-nat* promoter, ICER may modulate the threshold of cAMP-induced stimulation of MEL synthesis. This threshold would be fairly stable under typical L/D cycles but would alter under extreme photoperiodic cycles that affect ICER protein levels (Foulkes et al., 1996b; Li et al., 1998).

3. Sensitivity of the acute effect of NE on cAMP accumulation (Klein et al., 1981b) and AA-NAT activity (Deguchi and Axelrod, 1972b, 1973) increases 2- to 3-fold in rats kept in L/L, SCGx, or decentralized. This occurs gradually to reach a maximum after 7 days. This hypersensitivity probably results from an increase in β_1-AR density (Kebabian et al., 1975). In contrast, the sensitivity of the acute effect of NE on cGMP decreases by about 20-fold, to reach a minimum after 7 days (Klein et al., 1981b). Similarly, the activity of NOS decreases gradually by 80% to reach a minimum after 8 days of exposure of L/L or very long photoperiod (Schaad et al., 1994; Spessert et al., 1995; Jacobs et al., 1999). The decrease observed in L/L is prevented by daily injections of NE (Schaad et al., 1995a). It is therefore probable that repeated NAergic stimulation of NOS gene expression is responsible for the maintenance of cGMP sensitivity to NE. As expected, Spessert and Rapp (2001) also reported that the nocturnal peak of NOS mRNA displays photoperiodic variations (being longer in SP) leading to photoperiodic changes in protein expression of NOS type I.

4. Pineal AAAD activity is twofold higher in rats kept in L/L compared with D/D. SCGx provokes a similar effect (Snyder et al., 1965a). These observations suggest that NE regulates AAAD activity on a long-term basis, although probably through different mechanisms than those involved in the long-term regulation of HIOMT or NOS activity

B. Noradrenergic Regulation of Melatonin Synthesis in Other Mammalian Species

Regulation of the metabolic activity of the pineal gland in mammals other than the rat has been less well studied, partly because of inconvenience of use and partly because of the relative difficulty in stimulating MEL synthesis in some species.

1. Daily Regulation of Melatonin Synthesis

a. Daily Regulation of Melatonin Synthesis in Other Rodents. In the *Syrian hamster*, the nocturnal increase in MEL synthesis occurs late in the dark phase (Rollag et al., 1980; Miguez et al., 1995a). Daytime MEL values are approximately 0.2 ng/gland and increase up to 2 ng/gland at ZT 21 in LP (Miguez et al., 1995a). This nocturnal increase cannot be reproduced by acute or repeated β_1-AR stimulations during the day, but is inhibited by a β_1-AR antagonist given at night. In addition, an acute β_1-AR stimulation following a nighttime light exposure is able to reinduce MEL synthesis (Reiter et al., 1987). These data indicate that nocturnal stimulation of MEL synthesis is gated to the nighttime by unknown factors and results, at least partly, from an adrenergic input (Steinlechner et al., 1984b; Reiter et al., 1987). α_1-AR potentiation of β_1-AR stimulation has been reported (Nilsson and Reiter, 1989; Santana et al., 1989; Stankov et al., 1990b). The daily rhythm of MEL synthesis is driven by the nocturnal increase in the activity of AA-NAT that is, however, of a much less amplitude than that observed in the rat. HIOMT activity (around 97 ± 15 pmol/h/gland, n = 30) does not appear to vary in the course of the 24-h period (Steinlechner et al., 1984a; Ribelayga and Simonneaux, unpublished observations). The nocturnal increase in AA-NAT activity requires neo-transcription and neo-translation (Gonzalez-Brito et al., 1990). Indeed, *Aa-nat* mRNA level displays a large nocturnal increase (150-fold) peaking at ZT 20–22 (Gauer et al., 1999). Similarly to enzyme activity, *Aa-nat* mRNA could not be increased by acute or chronic injections of adrenergic agonists during the day but is inhibited at night following injection of a β- or α-adrenergic antagonist or to light exposure (Garidou et al., 2003a). Various experiments show that the pineal gland needs to be stimulated for at least 6 to 8 h in late afternoon to induce an increase in *Aa-nat*

mRNA, AA-NAT activity, and MEL synthesis (Gonzalez-Brito et al., 1988; Garidou et al., 2003a). The mechanisms controlling MEL synthesis in the Syrian hamster, therefore, appear somehow different to those described in the rat AA-NAT gene transcription, enzyme activation, and MEL synthesis during the night require the neosynthesis of a stimulatory protein (possibly transcription factor) but are repressed during the day by an inhibitory protein (possibly ICER), these processes leading to a strong gating of MEL synthesis in the late night (Diaz et al., 2003; Garidou et al., 2003a).

In the *Siberian hamster*, a large increase in the synthesis of MEL occurs at night (from undetectable levels during the day up to 0.7 ng/gland at ZT 16 in LP; Steinlechner et al., 1995; Ribelayga et al., 2000). There is a large nocturnal increase in AA-NAT activity (Illnerova et al., 1984) probably induced by *Aa-nat* gene transcription (Bernard et al., 1998). Light exposure or PROP injection at night induces a significant decrease in AA-NAT activity and MEL synthesis (Steinlechner et al., 1984b; Lerchl, 1995; Stieglitz et al., 1995). Injections of NE during the day stimulate AA-NAT activity and MEL synthesis within 3 to 4 h (Steinlechner et al., 1984b). The activity of HIOMT does not vary significantly over 24 h (Ribelayga et al., 2000). These observations indicate that NAergic activation of *Aa-nat* gene transcription and AA-NAT activity involve mechanisms similar to those described in the rat pineal gland.

In the *European hamster* a daily rhythm of MEL synthesis is observed throughout the year, although with marked seasonal variation in the length and amplitude of the nocturnal MEL peak (Pévet et al., 1989b; Vivien-Roels et al., 1992, 1997). As for other rodent species, the nocturnal increase in MEL synthesis depends on transcriptional activation of the *Aa-nat* gene (Garidou et al., 2003). The mechanisms involved in the regulation of MEL synthesis are not known. Nocturnal injection of PROP could partially inhibit nighttime levels of MEL. Acute or repeated injections of adrenergic agonists during the day, however, were not able to stimulate MEL synthesis, but a nighttime injection of a β_1-AR agonist was able to further increase the nocturnal level of MEL (Garidou et al., 2003). Therefore, MEL synthesis in the European hamster pineal gland is induced by NE but the stimulation is gated to the nighttime.

The production of MEL by the pineal gland of *mice* depends on the strains (Vivien-Roels et al., 1998; von Gall et al., 2000; Kennaway et al., 2002). Wild mice or few wild-derived inbred strains such as CBA or C3H produce significant amounts of MEL with a clear nocturnal increase, whereas most of the other inbred strains (C57black/J6, OF1 Swiss, BALB/c) have a low or undetectable level of pineal MEL with sometimes a very small and transient (15 min) nocturnal peak. Interestingly, MEL-deficient mice display a nocturnal peak of MEL, although with low amplitude, when they are raised under short photoperiod (von Gall et al., 2000). The inability to produce MEL does not occur in the early steps of the MEL biosynthesis pathway. NE induces equal increases in intracellular Ca^{2+}, P-CREB, and ICER in the pineal gland of MEL-proficient and MEL-deficient mice (von Gall et al., 2000). By contrast, activities of AA-NAT and HIOMT are elevated in wild or wild-derived mice (with a nocturnal increase of AA-NAT activity), whereas both are barely detectable in most other strains (Ebihara et al., 1987). In the C57black strain the *Aa-nat* gene was reported to include a 102-bp pseudoexon bearing a stop codon and giving rise to a severely truncated AA-NAT protein unable to synthesize MEL (Roseboom et al., 1998). Since most strains of mice display a clear day/night variation in *Aa-nat* gene transcription (Foulkes et al., 1996a; Roseboom et al., 1998), they may be used to study the regulation of *Aa-nat* transcription in a genetically modified mice model.

In contrast to the above-mentioned rodents, *Arvicanthis ansorgei* is a diurnal rodent (Challet et al., 2002). It was of interest therefore to check whether MEL synthesis in this species was similar to that observed in nocturnal rodents (Garidou et al., 2002). There is a marked increase (100-fold) in *Aa-nat* mRNA, which precedes that of AA-NAT activity and MEL by 2 h, both peaking 7 h after dark onset. These increases are partly reproduced by a daytime injection of a β_1-AR agonist. Toward the end of the night the decline of AA-NAT activity and MEL precedes that of *Aa-nat* mRNA, suggesting post-translational inhibition, as reported for the rat (Gastel et al., 1998). This is confirmed by the observation that 2 h after a nighttime injection of a β_1-AR antagonist the levels of AA-NAT activity and MEL content are reduced to daytime values, while *Aa-nat* mRNA levels are barely affected. Therefore, we found no fundamental differences between the nocturnal Wistar rat and diurnal *Arvicanthis ansorgei* in the mechanisms involved in NE-induced nocturnal stimulation of MEL synthesis.

b. Daily Regulation of Melatonin Synthesis in Nonrodents. In the *sheep*, stimulation of MEL synthesis depends on activation of a β_1-AR/cAMP/AA-NAT pathway (Morgan et al., 1988; Ravault et al., 1996). The role of α_1-AR stimulation has been discussed (Sugden et al., 1985a; Morgan et al., 1988; van Camp et al., 1991; Howell and Morgan, 1991). HIOMT activity (Namboodiri et al., 1985a,b) and *Hiomt* mRNA (Privat et al., 1999) do not vary significantly over the course of the daily 24 h cycle. Synthesis of MEL requires mainly translational and post-translational mechanisms. The level of *Aa-nat* mRNA is quite high during the day and increases by only 50% at night, whereas AA-NAT protein, enzyme activity, and MEL content are all low during the day and increase up to 10-fold during the night (Namboodiri et al., 1985a,b; Coon et al., 1995; Klein et al., 1997; Privat et al., 1999). Therefore, AA-NAT activation by cAMP requires synthesis of AA-NAT protein without de novo *Aa-nat* mRNA transcription (Klein et al., 1997). The high level of *Aa-nat* mRNA at the beginning of the night

results in a very fast increase (within a few minutes) in MEL synthesis immediately after the onset of darkness (Ravault et al., 1996; Ravault and Chesneau, 1999). It is probable that proteasome proteolysis is an important mechanism involved in the regulation of AA-NAT activity (see below for the cow).

In the *cow*, MEL synthesis occurs rapidly following onset of night (Hedlund et al., 1977). In vitro experiments showed that a β_1-AR stimulation elevates cAMP level, activates AA-NAY via a type II PKA, and increases MEL release (Ruppel and Olcese, 1991; Maronde et al., 1997; Schomerus et al., 2002). α_1-AR stimulation increases the intracellular level of Ca^{2+} in most pinealocytes but does not potentiate the β_1-AR-induced increase in cAMP level, AA-NAT activity, and MEL synthesis (Ruppel and Olcese, 1991; Schomerus et al., 2002). Increase in AA-NAT activity is blocked by puromycin, but not by actinomycin D (Chang and Ebadi, 1980). Regulation of AA-NAT activity was therefore proposed to result from translational and post-translational mechanisms, which was thereafter confirmed (Schomerus et al., 2000). Following cloning of the gene coding for bovine AA-NAT, it was shown that pineal *Aa-nat* mRNA levels are high both during the day and night with only a small increase at night (Craft et al., 1999). Recently, it was proposed that during the day, in the absence of cAMP, AA-NAT protein is constantly translated but instantly degraded by proteosomal proteolysis; in contrast, during the night, β_1-AR activation increases the levels of cAMP and PKA activity which, in turn, protects the protein from degradation and thereby enhances AA-NAT activity (Schomerus et al., 2000).

In *humans* and *monkeys* limited studies suggest a "sheep-like" regulation. There is an immediate increase in circulating melatonin at the onset of darkness (Reppert et al., 1979; Arendt, 1995). In rhesus monkey and human, the quantity of *Aa-nat* mRNA is high and displays no daily variations, while the enzyme activity increases by up to 10-fold at night (Coon et al., 1996, 2002). The mean daily level of pineal HIOMT activity is about 4.3 ± 0.1 nmol/h/mg protein in human (Bernard et al., 1995) and about 9 nmol/h/mg protein in rhesus monkey (Coon et al., 2002) with no significant day/night variation. Daytime β_1-AR stimulation does not stimulate MEL synthesis (Berlin et al., 1995), but its nocturnal synthesis can be inhibited by a β_1-AR antagonist (Cowen et al., 1985). In humans, there is a large interindividual variability in the daily pattern of MEL synthesis, which also varies depending on age (Baskett et al., 2001).

c. Conclusions. Studies performed so far in different mammalian models show that the nocturnal increase in MEL synthesis is primarily triggered by an increase in AA-NAT activity resulting from accumulation of the AA-NAT protein itself. Nevertheless, fundamental differences in the mechanisms involved in the accumulation of stable and active AA-NAT molecules exist (Fig. 7). Two groups of mammals can be distinguished: first, the rodent species ("rat type"), in which an increase in the expression of the *Aa-nat* gene and synthesis of new AA-NAT molecules are a requirement, and secondly the nonrodent species ("sheep-type"), in which *Aa-nat* mRNA is constitutively present at a high level and AA-NAT protein accumulation results basically from stabilization of the constantly translated protein. These different mechanisms are responsible for the different patterns of MEL synthesis and secretion observed between the two groups (see Klein et al., 1997; Stehle et al., 2001 for reviews) with a long delay (several hours) from dark onset to MEL onset in rodents and a very short delay (a few minutes) from dark onset to MEL onset in nonrodents.

Unfortunately, there have been far fewer biochemical and molecular studies performed in the above species compared to the rat. Analyses of these findings, however, show that, although NE is probably an important neurotransmitter regulating daily MEL synthesis, most of these species are not fully responsive to NE, suggesting the involvement of other transmitters to obtain a full MEL response.

2. Seasonal Variations in Melatonin Synthesis

a. Variations in the Duration of the Nocturnal Melatonin Peak. In most mammalian species studied so far, an increase in the duration of the dark phase results in a lengthening of the duration of the nocturnal MEL peak up to a maximum, which differs according to species. In addition, the characteristics of the lengthening of the nocturnal peak are different according to species (see Pévet et al., 1991; Reiter, 1993; Pévet and Pitrosky, 1997 for reviews). For example, in the *rat* at the beginning of the night, the time between dark onset and MEL onset increases when the night duration lengthens, whereas at the end of the night the decline in MEL secretion occurs shortly before light onset (initiated by the circadian clock), irrespective of the photoperiod (Illnerova and Vanecek, 1980). This MEL rhythm is driven by photoperiodic variations in the duration of the nocturnal peak of *Aa-nat* mRNA and activity (Illnerova and Vanecek, 1980; Illnerova, 1986; Ribelayga et al., 1999a). The consequence of this regulation is an increase in the duration of the nocturnal MEL peak until it reaches a maximum, after which lengthening the night results in no further increase in the MEL peak duration. In the *Siberian hamster*, photoperiodic regulation of AA-NAT activity and MEL synthesis is similar to that of the rat except that the decrease in MEL release at the end of the night is initiated by morning light in LP and probably by the circadian clock in SP (Illnerova et al., 1984). In the *Syrian hamster*, the increase in MEL synthesis at the beginning of the night occurs after dark onset with the same delay whatever the photoperiod. The decline in MEL production at the end of the night is initiated by the light in LP and probably by the circadian clock in SP (Skene et al., 1987; Miguez et al., 1995a). In the *European hamster*, in contrast to the rat, the delay between

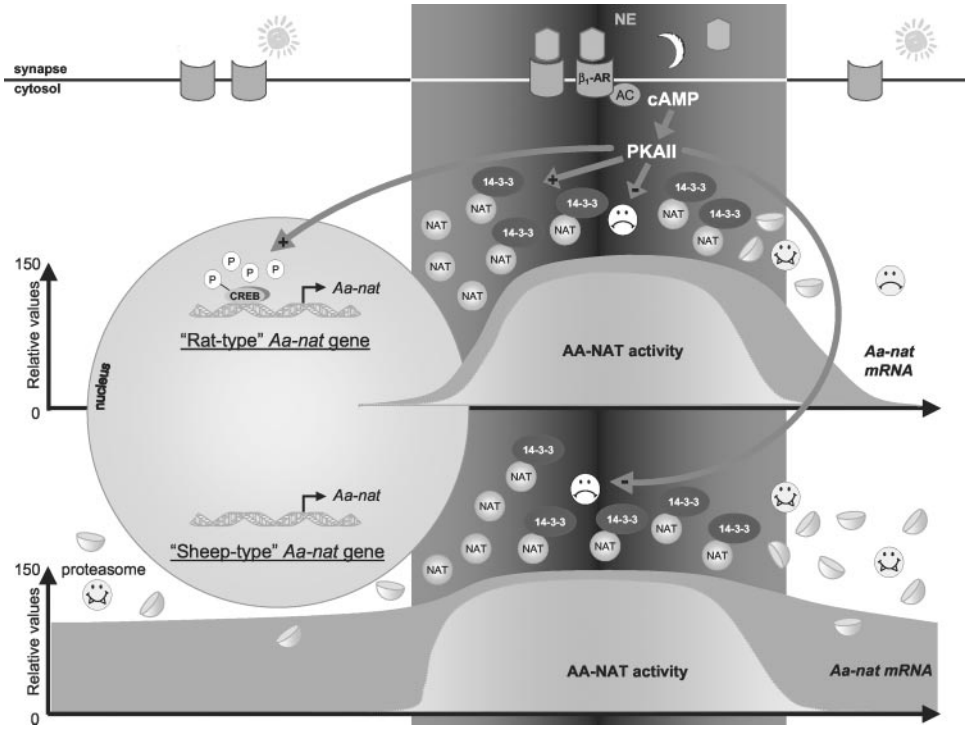

FIG. 7. Schematic representation of the two types of regulation of AA-NAT activity described in the mammalian pineal gland. In the pineal gland of all mammals studied to date, AA-NAT activity increases during the night as a result of the nocturnal adrenergic stimulation. However, two groups of mammals can be distinguished on the basis of the molecular mechanisms leading to stimulation of AA-NAT activity. In a group comprising many rodent species, the nocturnal increase in AA-NAT activity results firstly from the cAMP/PKA-dependent stimulation of *Aa-nat* gene expression (100–150-fold) with the subsequent synthesis of new molecules of AA-NAT. Additionally, cAMP/PKA phosphorylates AA-NAT, which allows its interaction with a chaperone protein 14-3-3 and inhibits proteasomal proteolysis of the AA-NAT molecules. During the day, AA-NAT activity is relatively low because of the low basal expression of the *Aa-nat* gene and low levels of the proteolysis inhibitor. In a second group of mammals including ungulates (e.g., sheep, cattle) and apes, the *Aa-nat* gene is constitutively expressed and the level of *Aa-nat* mRNA displays small (if any) daily variations. However, even though *Aa-nat* mRNA is continuously translated, the AA-NAT protein only accumulates during the night when NE-induced accumulation of cAMP prevents proteasomal proteolysis of AA-NAT molecules. In addition, cAMP/PKA activates AA-NAT following phosphorylation and interaction with the 14-3-3 proteins. The presence of a readily available pool of *Aa-nat* mRNA at the beginning of the night accounts for the rapid increase in MEL synthesis immediately after lights-off. During the day, in the absence of cAMP, the AA-NAT molecules are continuously lysed as soon as they are synthesized, thus accounting for the low daytime level of AA-NAT activity.

dark onset and MEL onset is shorter when the night duration lengthens. As in other photoperiodic species, the decrease in MEL synthesis and release at the end of the night is initiated by light when animals are kept in LP. Such regulation results in large photoperiodic variations in MEL duration (Vivien-Roels et al., 1997; Garidou et al., 2003b).

b. Variations in the Amplitude of the Nocturnal Melatonin Peak. In addition to photoperiodic variations in the duration of the nocturnal MEL peak, a seasonal variation in the amplitude of this peak is also observed in certain species. In the *European hamster*, raised in natural conditions, the daily rhythms in MEL and 5-ML synthesis display marked seasonal variations. The amplitude of the nocturnal MEL peak is high from September to April (with a maximum of 10-fold nocturnal increase around November/December) and very low during the summer (with a minimum of a 1.5-fold increase in June; Vivien-Roels et al., 1992, 1997). Interestingly, this photoperiodic variation in MEL peak amplitude is driven by *Aa-nat* mRNA and AA-NAT activity levels (Garidou et al., 2003b). Similarly, the diurnal levels of 5-ML are the highest in autumn/winter (Vivien-Roels et al., 1992). In addition, we have observed a seasonal variation in HIOMT activity, with an increase in late autumn associated with an increase in MEL and 5-ML synthesis, suggesting that this enzyme is also involved in the seasonal regulation of pineal metabolism in the European hamster (Ribelayga et al., 1998c). Understanding the underlying mechanisms involved in this seasonal regulation is difficult because this species is endowed with an endogenous circannual clock (Masson-Pévet et al., 1994b; Saboureau et al., 1999), and the amount of MEL synthesis appears to be modulated by the external temperature, with lower temperature increasing the MEL peak amplitude (Vivien-Roels et al., 1997). Interestingly, we observed that administration of a β-adrenergic agonist during the night in LP augments the low nocturnal level of MEL up to values observed at night in SP (Garidou et al., 2003b). This suggests that the low amplitude of the MEL peak in LP results from a weaker NEergic input from the circadian clock toward the pineal gland. In the *Siberian hamster*, several studies have reported that the amplitude of the nocturnal MEL peak is 2-fold higher in animals raised in SP than in LP (Illnerova et al., 1984; Hoffmann et al., 1985;

Lerchl and Schlatt, 1992; Miguez et al., 1996; Ribelayga et al., 2000) and in winter compared to summer (Steinlechner et al., 1995). These variations do not result from an increase in the amplitude of the peak of AA-NAT activity since, in contrast, this amplitude is about 2-fold lower in SP compared to LP (Hoffmann, 1981; Illnerova et al., 1984; Ribelayga et al., 2000). Similar observations have also been made in natural conditions, an annual rhythm in the amplitude of the nocturnal AA-NAT activity peak has been demonstrated with a maximum in summer and a minimum in winter (Steinlechner et al., 1987). In contrast to AA-NAT activity, the mean daily level of HIOMT activity is about 2-fold higher under SP compared to LP, without modification of the enzyme affinity for its substrates, indicating that this increase results from an increase in the amount of protein (Ribelayga et al., 2000). These results demonstrate that in some photoperiodic species, photoperiodic variations in HIOMT activity drive the photoperiodic variations in the amplitude of the nocturnal MEL peak. Studies have now to be performed to understand the mechanisms involved in the photoperiodic regulation of HIOMT and its role in the seasonal regulation of MEL.

c. Conclusions. Physiologically, the seasonal variations in MEL synthesis and release confer the major function of the mammalian pineal gland that is to synchronize annual functions with seasons. However, while the basic mechanisms involved in the daily regulation of MEL synthesis have been actively investigated, especially in the (nonphotoperiodic) rat, the mechanisms underlying the photoperiodic/seasonal variations in MEL synthesis are less well known. In most photoperiodic species it is clear that NE alone is not sufficient to fully stimulate MEL synthesis, thus revealing an important role for other pineal transmitters. Although photoperiodic regulation of MEL synthesis is probably primarily driven by photoperiodic alterations in the hypothalamic SCN clock activity, further studies are clearly needed to elucidate the photoperiodic regulation of NE and other neurotransmitters that allow decoding of the photoperiodic message by the pineal gland.

C. Conclusion: Both AA-NAT and HIOMT Shape the Daily and Seasonal Profiles in Melatonin Synthesis

In the pineal gland of most mammals, the nocturnal increase in MEL synthesis and release is primarily driven by AA-NAT activity. Studies on the regulation of this enzyme in the rat have shown that the release of NE at the beginning of the night activates both β_1- and α_1-AR, resulting in a large increase in the intracellular levels of cAMP and PKA-induced phosphorylation of CREB into P-CREB. The latter transcription factor is thought to induce (at least partly) a massive expression ($\times$150) of the gene coding for AA-NAT. The enzyme, rapidly synthesized/activated ($\times$50–70), catalyzes the synthesis of MEL from 5-HT. NAergic stimulation also induces, but to a lesser degree, the expression of genes coding for TPOH ($\times$1.5) and HIOMT ($\times$2), and other transcription factors that do not appear to be involved in the nocturnal stimulation of MEL synthesis but rather in the modulation of this stimulation. Cessation of NE release at the end of the night or following a light exposure results in a rapid decrease in cAMP levels followed by post-translational inhibition of AA-NAT activity (destabilization/proteolysis). In nonrodent species, nocturnal increase in the synthesis of MEL appears to depend mainly on post-translational mechanisms (see Klein et al., 1997; Stehle et al., 2001 for reviews). The high level of *Aa-nat* mRNA throughout the 24-h cycle allows a sustained synthesis of AA-NAT protein that is rapidly degraded by proteasome proteolysis during the day, whereas at night NE-induced cAMP accumulation inhibits AA-NAT proteolysis and allows rapid enzyme activation and MEL synthesis.

Besides *Aa-nat*, *Hiomt* mRNA is also regulated every night by the NE input, but with a different effect of time on HIOMT activity, due to the much higher stability of HIOMT protein compared to AA-NAT. Consequently, HIOMT activity displays a significant photoperiodic/seasonal variation in the pineal gland of several rodent species, with a higher activity under longer nights (Ribelayga et al., 1998c, 1999a, 2000). As shown in the Siberian hamster, HIOMT activity may be the limiting factor for the rate of MEL synthesis at night, and therefore the photoperiodic variation in HIOMT activity may drive the photoperiodic variation in the amplitude of the MEL peak.

We therefore propose that AA-NAT and HIOMT are both involved in the regulation of the MEL message but with rather different functions (Fig. 8): AA-NAT switching MEL synthesis on and off (with photoperiodic variations in duration) and HIOMT tuning the amplitude of this nocturnal MEL synthesis (with photoperiodic variation in magnitude).

VI. Regulation of Melatonin synthesis in the Mammalian Pineal Gland by Other Transmitters

The function of the pineal hormone MEL is unusual because it depends on the pattern of its secretion (namely the duration and amplitude of the nocturnal peak, and coincidence of this secretion with target sensitivity). This is why regulation of its synthesis and release probably requires a complex control, as the presence of many transmitters and their receptors in the pineal gland suggests.

While, as summarized above (see *Section V*), the presence of NAergic fibers, as well as the role and mechanisms of action of NE, have been well studied for more than 30 years, the role of the other pineal transmitters is now emerging.

In 1984, Ebadi began his review on the regulation of MEL synthesis by writing that "a pinealogist should view a pinealocyte as containing numerous and cascad-

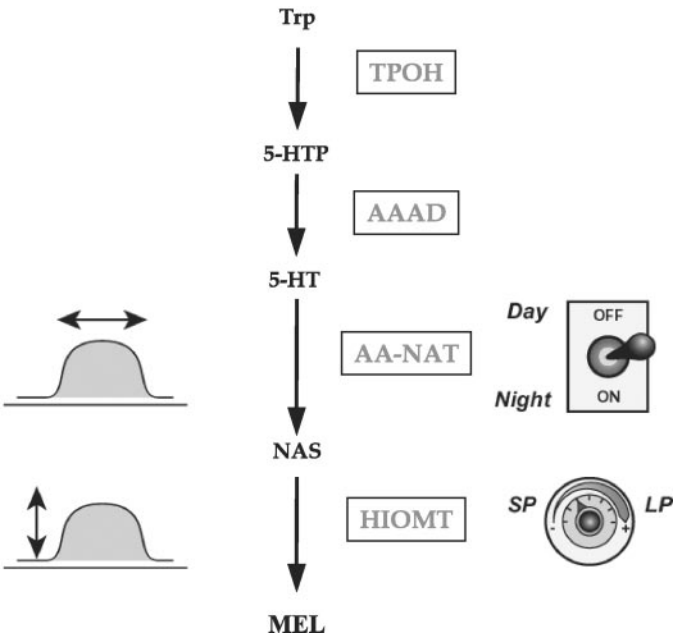

FIG. 8. Schematic representation of the different roles of AA-NAT and HIOMT in the daily and photoperiodic regulation of MEL synthesis. The marked onset of AA-NAT activity at the beginning of the night and its offset later in the night drives the duration of the nocturnal MEL peak, whereas photoperiodic variations of HIOMT activity, with lower values under LP, drives the amplitude of the nocturnal MEL peak. This functional hypothesis is to be adapted according to species (drawing from F. Revel, unpublished report).

ing groups of receptor sites, one of which is a β-adrenergic site (. . .), view the pinealocyte as containing and orchestrating the functions of numerous neurotransmitters, one of which is norepinephrine (. . .), remain cognizant of the remarkable species-directed specificity of the mammalian pineal gland in synthesizing MEL." Seventeen years later, this introduction to the control of MEL production in mammals remains truer than ever.

A. Peptidergic Regulation of Melatonin Synthesis

Pévet (1981, 1983b, 1986) was one of the first authors to point out the large variety of peptides contained in the mammalian pineal gland. Before this, studies had focused on the search for a specific pineal peptide with antigonadotropic properties. In the early 1980s, the hypothesis that pineal metabolic activity may be regulated by peptides was introduced. Since then, several research groups have been seeking to determine the origin, sites of action, effects, and physiological roles of the (neuro)peptides in the mammalian pineal gland.

The mammalian pineal gland contains a great diversity of peptides of different origins (Pévet, 1983b): nervous fibers (neuropeptides) of sympathetic, central, or parasympathetic origin; systemic circulation (peptidergic hormones); and cells of the pineal itself releasing peptides with autocrine/paracrine effects. Studies on pineal peptides and their relation to MEL synthesis have been the object of previous reviews (Vaughan, 1984; Pévet, 1986; Ebadi et al., 1989; Møller et al., 1991b; Møller, 1994, 1999; Simonneaux, 1995; Simonneaux et al., 1996b; Simonneaux and Pévet, 1998). Since then, a lot of experimental data have come to support the hypothesis of a significant physiological role of peptidergic regulation on mammalian pineal metabolism.

1. Vasoactive Intestinal Peptide, Pituitary Adenylate Cyclase Activating Peptide, and Histidine Isoleucine Peptide. The neuropeptides VIP, PHI, and PACAP belong to the VIP/secretin/glucagon family and display a remarkable amino acid sequence homology because they originate from a single ancestral molecule, probably PACAP itself (see Sherwood et al., 2000; Vaudry et al., 2000 for reviews). VIP is a 28-amino acid peptide, isolated for the first time by Said and Mutt in 1970 from the porcine gut, and then identified in many central (especially in the cortex, hippocampus, hypothalamic nuclei, amygdala) and peripheral nervous structures. PHI is a 27-amino acid peptide, originating from the *VIP* precursor-coding gene, which was isolated in 1980 by Tatemoto and Mutt. PACAP is a 38-amino acid peptide that occurs, although to a lesser extent, in a shorter form of a 27-amino acid peptide curtailed in position C-terminal. It was isolated for the first time by Miyata et al. (1989) from the sheep hypothalamus and further described as an important neuropeptide of the central nervous system and peripheral organs. It is particularly abundant in the adrenal gland, testis, pituitary, and various brain regions such as the thalamic and hypothalamic nuclei, lateral septum, and dorsal raphe nuclei.

VIP is involved in vaso- and bronco-dilation, in the regulation of the synthesis and secretion of several hormones (prolactin and growth hormone) and body fluids (saliva), in neuronal growth and survival, in neurotransmission, and in immunity (Said, 1991; Nussdorfer and Malendowicz, 1998). PACAP exerts pleiotropic functions: it is involved in cell survival, differentiation, proliferation and apoptosis, in spermatogenesis, in the regulation of synthesis and release of various hormones (from the pituitary, adrenal gland, pancreas) and in neurotransmission (Rawlings and Hezareh, 1996; Sherwood et al., 2000; Vaudry et al., 2000). Recent data have shown that both peptides are involved in the regulation and the expression of circadian rhythms. VIP is present in the SCN neurons with a day/night variation in its content and may be part of the endogenous clock output (Ibata et al., 1989; Shinohara et al., 1994). Since the type 2 VIP/PACAP ($VPAC_2$-R) is also expressed in the SCN, VIP may also exert phase-resetting properties (Piggins et al., 1995; Reed et al., 2001). PACAP also recently appeared as an important neurotransmitter of the circadian system (Hannibal et al., 1997, 2000, 2001; Kopp et al., 1997). It is present in the RHT, colocalized with Glu, and is able to induce phase-shifting of the circadian clock either during the subjective day or subjective night using cAMP-dependent or Ca^{2+}-dependent mechanisms, respectively (Hannibal et al., 1997; Harrington et al., 1999; Kopp et al., 1999). Finally, we report below that

these peptides are involved in the regulation of pineal MEL synthesis and release.

The pineal gland of all mammalian species studied so far contains a VIPergic innervation (see Cozzi, 1999 for review). The species studied include rabbit, cat and pig (Uddman et al., 1980), rat (Mikkelsen et al., 1987), gerbil (Møller et al., 1985; Shiotani et al., 1986), sheep (Cozzi et al., 1990), and mouse (Mikkelsen et al., 1994). The concentration of VIP in the rat pineal gland is 17 pmol/g (Møller and Mikkelsen, 1989). SCGx does not alter VIPergic innervation in the pineal gland of the rat (Møller and Mikkelsen, 1989; Piszczkiewicz and Zigmond, 1992) and sheep (Cozzi et al., 1994), indicating that the VIP fibers are of extra-sympathetic origin. Shiotani et al. (1986) have demonstrated, in the gerbil, that the VIP fibers originate from the parasympathetic pterygopalatine ganglia. In addition, some VIP fibers may originate from central structures that project to the pineal gland (Møller et al., 1985) or from the trigeminal ganglia (in the sheep, Cozzi, 1999). VIPergic fibers of parasympathetic origin enter the pineal gland through the pial capsule, travel within the gland following the blood vessels, and end among clusters of pinealocytes; central VIPergic fibers enter via the deep pineal gland. Some VIPergic nerve endings are found in the perivascular space, which suggests a vasorelaxant effect on pineal blood flow (Nilsson, 1994). In sheep, VIPergic fibers contain the neuronal type NOS (Lopez-Figueroa and Møller, 1996; Lopez-Figueroa et al., 1996). In some parasympathetic structures, NO can regulate neurotransmitter release (Modin et al., 1994). In the rat, the presence of NOS-containing fibers has not been demonstrated but it is suggested by the report of colocalization of NOS with VIP and PHI in the pterygopalatine ganglia (Ceccatelli et al., 1994). PHI is present in the VIPergic fibers innervating the pineal gland of the rat (Møller and Mikkelsen, 1989), sheep (Cozzi et al., 1994), and mouse (Mikkelsen et al., 1994). PACAP is present in structures whose neurons project to the pineal gland (PVN: Masuo et al., 1993; SCG: Klimaschewski et al., 1996a; trigeminal ganglia: Møller et al., 1993, 1999). PACAPergic fibers were observed in the pineal gland of rat (Liu and Møller, 2000), sheep (Liu et al., 2000), and pig (Nowicki et al., 2002). They originate from the trigeminal ganglia and reach the pineal gland via the conarian nerve (Liu and Møller, 2000; Liu et al., 2000).

VIP and PACAP have a similar structure, with 68% of homology. VIP was the first and most studied neuropeptide in the pineal gland. VIP increases the intracellular levels (Kaneko et al., 1980; Yuwiler, 1983a; Simonneaux et al., 1997b) and efflux (Rekasi et al., 1998) of cAMP and therefore activates all cAMP-related events: phosphorylation of CREB (Roseboom and Klein, 1995; Schomerus et al., 1996), increase in *Aa-nat* gene expression (Roseboom et al., 1996; Rekasi and Czompoly, 2002), activation of AA-NAT activity in vitro (Kaneko et al., 1980; Yuwiler, 1983a) and in vivo (Schröder et al., 1989), stimulation of the synthesis and release of 5-HT probably following TPOH activation (Simonneaux et al., 1997c), long-term activation of HIOMT (Ribelayga et al., 1997), and stimulation of MEL release (Simonneaux et al., 1990c, 1993). These effects, however, are always lower than what has been reported following β-AR stimulation. Surprisingly, VIP has also been reported to increase cGMP levels (Ho et al., 1987b) by NO-dependent mechanisms (Spessert, 1993) and the influx of extracellular Ca^{2+} through cGMP-sensitive Ca^{2+} channels (Schaad et al., 1995b). It should be noted, however, that other authors have not observed an effect of VIP on $Ca^{2+}{}_i$ (Olcese et al., 1996; Schomerus et al., 1996). The stimulatory effect of VIP on cAMP, cGMP, and AA-NAT is potentiated by α_1-AR agonists (Ho et al., 1987b; Yuwiler, 1987; Chik et al., 1988). However, it has been suggested that, in addition to its postsynaptic effects on pinealocytes, VIP may stimulate TH activity in the sympathetic nerve endings (Schwarzschild and Zigmond, 1991). A study comparing the effects of VIP and ISO on the same culture of rat pinealocytes has shown that VIP is very effective in stimulating MEL synthesis (EC_{50} = 0.11 nM), but that at optimal doses (1 to 10 nM) its effect is approximately 2 to 3 times lower than that induced by optimal doses (1 to 10 μM) of a β_1-AR agonist (Simonneaux et al., 1993). These observations are reinforced by the data of Schomerus et al. (1996) showing that VIP induces CREB phosphorylation in 50 to 60% of cultured pinealocytes whereas NE induces it in 95% of pinealocytes. It appears, therefore, that only about half of the rat pinealocytes are endowed with VIP binding sites, while nearly all contain β_1-AR. The publication of a study in 1993 showing the presence of PACAP in the rat pineal gland (Masuo et al., 1993) led us to study the effect of this peptide. PACAP stimulates the synthesis and release of MEL by cultured rat pinealocytes with a high affinity (EC_{50} = 0.14 nM) similar to that of VIP (Simonneaux et al., 1993). PACAP, like VIP, increases CREB phosphorylation (Schomerus et al., 1996), cAMP accumulation (Chik and Ho, 1995; Simonneaux et al., 1997b), *Aa-nat* gene expression (Rekasi and Czompoly, 2002), AA-NAT activity (Yuwiler et al., 1995), 5-HT synthesis (Simonneaux et al., 1997c), and the long-term activity of HIOMT (Ribelayga et al., 1997). Intensity of these PACAP effects is similar to that of VIP (thus lower than reported following NE stimulation). The effect of PACAP on cAMP and AA-NAT may be potentiated by α_1-AR stimulation (Chik and Ho, 1995; Yuwiler et al., 1995). PACAP increases the concentration of $Ca^{2+}{}_i$ (Olcese et al., 1996; Simonneaux, unpublished observations, but see Schomerus et al., 1996). Interestingly, PACAP, in contrast to VIP, does not stimulate cGMP accumulation in the rat pineal gland (Chik and Ho, 1995).

The reported qualitative and quantitative similarities between VIP and PACAP prompted us to investigate whether these two peptides act on similar or different

receptors. There are three types of receptors for VIP and PACAP (IUPHAR nomenclature: Harmar et al., 1998): the PACAP specific receptor (PAC_1-R) displays a higher affinity (100 to 1000 times) for PACAP than for VIP; VIP_1/PACAP ($VPAC_1$-R) and VIP_2/PACAP ($VPAC_2$-R) receptors show a similar affinity for VIP and PACAP. The PAC_1-R is coded by a gene that may be expressed under six different splice variants (with or without different combinations of two cassettes of 81 (*hop1* or *hop2*) and 88 (*hip*) nucleotides) (Spengler et al., 1993). In addition, a very short form (amputated of 21 amino acids in the extracellular *N*-terminal portion of the protein) has been observed (Pantaloni et al., 1996). All PAC_1-R variants activate AC with equal potency but induce PLC activity to varying degrees according to the splice variant. Finally, an eighth variant of PAC_1-R with amino acid substitutions and deletions in the second and fourth transmembrane domains (PAC_1-*R*-TM4) has been cloned and reported to affect an L-type Ca^{2+}-channel with no effect on AC and PLC activities (Chatterjee et al., 1996). $VPAC_1$-R and $VPAC_2$-R are coded by two different genes (*$VPAC_1$-R*: Ishihara et al., 1992; *$VPAC_2$-R*: Lutz et al., 1993) without known alternative splicing; they mainly differentiate by their relative affinities for secretin (lower for $VPAC_2$-R). Activation of these receptors always induces an increase in cAMP levels. Originally activation of these receptors was thought not to affect the IP_3/PLC system; however, there are a few examples where the VPAC-R might increase inositol phosphate production or affect Ca^{2+} levels.

In 1983, Kaku et al. reported the presence of VIP binding sites in the rat pineal gland. However, recent data on the effects of PACAP in the pineal gland, the existence of several types of VIP/PACAP receptors, and the development of specific agonists/antagonists for these receptors (Gourlet et al., 1997a,b,c) have allowed us to characterize the nature of the binding sites of these peptides in the rat pineal gland. We have demonstrated by ligand binding experiments, RT-PCR analysis, pharmacological, and biochemical analyses that VIP and PACAP bind equally to the $VPAC_1$-R to stimulate the MEL synthesis via a cAMP-dependent mechanism (Simonneaux et al., 1997b). The presence of PAC_1-R in the rat pineal gland is being questioned since we observed by RT-PCR that the short and *hop* splice variants of PAC_1-R are expressed in the rat pineal gland (Simonneaux et al., 1997b) but no specific labeling for the gene coding for PAC_1-R was observed by ISH in the rat pineal gland (Hashimoto et al., 1996). Nevertheless, if the PAC_1-R is present and functional in the rat pineal gland, it does not appear to be involved in the stimulation of MEL synthesis. On the one hand, the stimulatory effects of VIP and PACAP on MEL synthesis are not additive, and on the other hand a $VPAC_1$-R antagonist inhibits the effect of VIP (EC_{50} approximately 19 nM) and PACAP (EC_{50} approximately 37 nM) with a similar affinity on MEL secretion (Simonneaux et al., 1997b). The PAC_1-R, if expressed in the pineal gland, could regulate other functions, for example blood pressure (Nilsson, 1994) or the synthesis and release of NE and NPY from nerve terminals (May and Braas, 1995). The role of PAC_1-R is therefore still to be established in the pineal gland. In addition, it will be necessary first to define whether VIP and PACAP display different effects on $Ca^{2+}{}_i$; secondly to establish whether PACAP, but not VIP, increases IP_3, this effect being specific to PAC_1-R; and thirdly to define whether the effect of VIP on cGMP, not observed with PACAP (Chik and Ho, 1995), is a phenomenon induced by $VPAC_1$-R or by another type of VIP receptor.

The rat pineal gland contains two other peptides belonging to the same family: helodermin and PHI. Helodermin increases cAMP levels and AA-NAT activity similarly to VIP (EC_{50} = 1 nM) and it is possible that it acts on VIP receptors known to display a high affinity for helodermin (Kaku et al., 1992). PHI stimulates AA-NAT activity and MEL synthesis similarly to VIP (Moujir et al., 1992). Binding sites for PHI have been described in the rat pineal gland (Tsuchiya et al., 1987); however, it is very probable that PHI also binds VIP receptors (Sherwood et al., 2000).

The presence, sites of action, and effects of these peptides have been poorly studied in other mammalian species. VIP stimulates MEL synthesis in the sheep (Morgan et al., 1988) but not in the Syrian hamster (Moujir et al., 1992). Contradictory data were reported in the bovine pineal gland since high-affinity VIP binding sites (K_D = 5 nM) (Samejima et al., 1993) but no mRNA coding for PAC_1-R (Olcese et al., 1996) were reported, whereas PACAP, but not VIP, was found to slightly increase cAMP level, AA-NAT activity, and MEL release in cultured bovine pineal cells (Schomerus et al., 2002).

The presence of VIP and PACAP in the pineal gland of mammals and the demonstration in the rat of their powerful stimulatory effect on the cAMP/AA-NAT/MEL pathway via the activation of $VPAC_1$-R (Fig. 9A) suggests that they are important neuromodulators of MEL synthesis. Their maximal stimulation of MEL release in vitro is always 2 to 5 times lower than that obtained after maximal NAergic stimulation. However, optimal VIP (or PACAP) concentrations are able to further increase MEL release induced by suboptimal concentrations of ISO (Simonneaux et al., 1997b; Fig. 9B). This observation is of special interest since the VIP content of the rat pineal gland displays a 3-fold nocturnal increase (Kaku et al., 1986; Fig. 9C). In addition, the effect of VIP is modulated by light (Yuwiler, 1983b; Kaku et al., 1985), suggesting a role for VIP in the transmission of photic information to the pineal gland. The content of PACAP in the rat pineal gland increases 2-fold at night (Fukuhara et al., 1998), although this is controversial (Møller et al., 1999). The occurrence of seasonal variations in pineal VIP or PACAP content has not been reported to date.

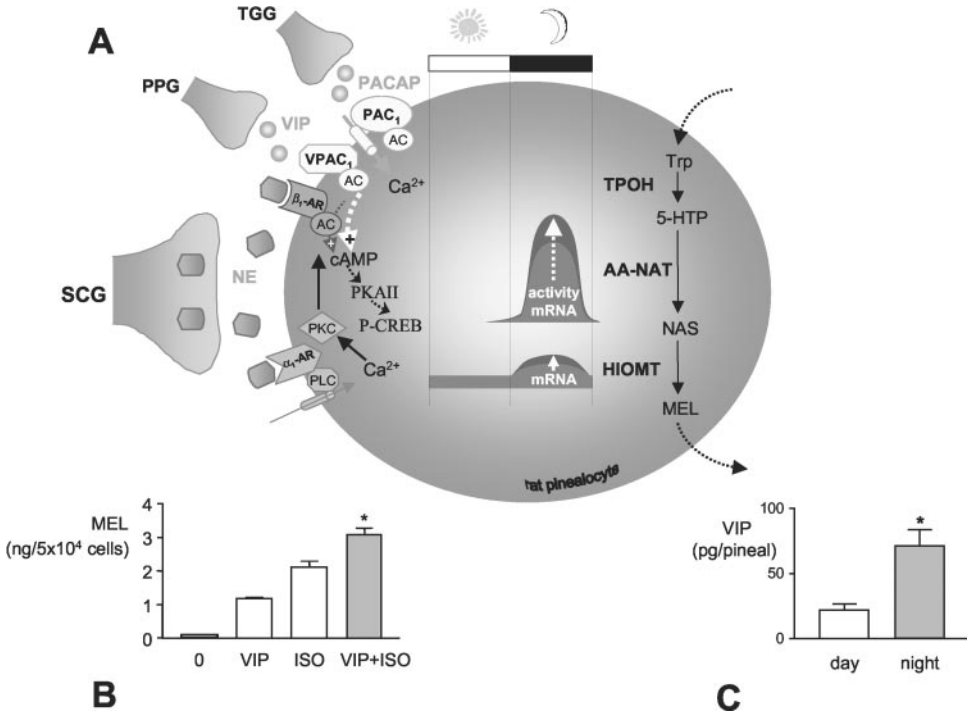

FIG. 9. A, intracellular effects of VIP and PACAP on the MEL synthesis pathway in rat pinealocytes. VIP (mainly originating from the PPG) and PACAP (mainly originating from the TGG) bind to $VPAC_1$-R to activate the cAMP/PKA/P-CREB pathway and increase AA-NAT mRNA, AA-NAT activity, and MEL release. PACAP binding to PAC_1-R increases Ca^{2+} levels. B, VIP stimulation of MEL release from cultured rat pinealocytes may be additive to that induced by the β-AR agonist isoproterenol (ISO). Dissociated rat pineal cells were cultured for 48 h in a standard culture medium and incubated for 5 h with VIP (10 nM) and/or ISO (100 nM). MEL was measured in the culture medium by radioimmunoassay. ⋆, $P < 0.05$ compared to other values. C, VIP content in the rat pineal gland is higher at night than during the day. Rats were sacrificed during the day (12:00) or night (4:00) and VIP was measured in the pineal gland by radioimmunoassay; ⋆, $P < 0.05$ compared to daytime values (modified from Kaku et al., 1986, with permission).

The above observations show that VIP and PACAP are present in nerve fibers of the pineal gland, display daily variations, directly stimulate, and further increase β_1-AR stimulation of MEL synthesis with alterations depending on the light environment. These findings strongly suggest their involvement in the nocturnal secretion of MEL, although experimental models still have to be designed to test this hypothesis.

2. Neuropeptide Y. NPY is a 36-amino acid peptide rich in tyrosine. It was isolated for the first time by Tatemoto et al. (1982) from porcine brain. It was later described as one of the neuropeptides whose concentration is the highest in the central and peripheral nervous system (see Larhammar, 1996; Malendowicz et al., 1996 for review). It is present in the limbic structures, cortex, hypothalamus, cerebral trunk, spinal cord, and vascular bed of many organs. NPY belongs to the pancreatic polypeptide family (NPY, YY peptide (PYY), and pancreatic peptide (PP)), all members with a large number of Y residues including both ends of the molecule, sharing a high amino acid homology, and characterized by a hairpin tertiary structure. However, while NPY acts as a neurotransmitter, PYY (mainly present in the intestine endocrine cells) and PP (mainly present in pancreatic cells) act as hormones. NPY is often associated with the sympathetic nervous system, where it is colocalized with NE, but it is also present in neurons of the central nervous system. Two main functions are attributed to NPY: 1) regulation of NAergic transmission pre and postsynaptically (especially in the vascular system of various organs, where it has a vasoconstrictor effect); and 2) control of food intake, since it appears to be a powerful stimulator of food and water intake. It is also involved in the regulation of learning, the regulation of the secretion of several hormones (VP, OT, corticosterone, αMSH, LHRH), the control of body temperature, and in epilepsy. NPY, originating from the thalamic IGL, is also an important input to the SCN, where it is reported to alter the phase of the endogenous circadian oscillator. Interestingly, it displays a nonphotic-like effect during the subjective day via presynaptic Y_2-R and inhibits photic phase shifting during the subjective night via postsynaptic Y_5-R, and maybe Y_1-R (see Gribkoff et al., 1998; Yannielli and Harrington, 2001; for reviews). In addition, we report below that NPY is a pineal neurotransmitter regulating the synthesis of MEL.

NPY binds to several receptors (Y_n-R) all belonging to the superfamily of G-protein-coupled receptors. These receptors were first described as belonging to two types: Y_1-R, mainly present postsynaptically, and Y_2-R, present presynaptically (Wahlestedt et al., 1986). Following cloning of the gene coding for Y_1-R (Herzog et al., 1992) and then for Y_2-R (Rose et al., 1995), other types of

Y_n-R were identified (see Larhammar, 1996; Michel et al., 1998 for reviews): Y_3-R (Herzog et al., 1993), Y_4-R (Lundell et al., 1996), Y_5-R (Hu et al., 1996; Haynes et al., 1998, the "food intake" receptor), and Y_6-R (Weinberg et al., 1996). In addition, it is noteworthy that several studies have suggested that NPY may be one of the endogenous ligands of σ receptors (Roman et al., 1989). To date, a few agonists have been found to differentiate Y_1-R, Y_2-R, and Y_3-R (Fuhlendorff et al., 1990), but only one specific nonpeptidic Y_1-R antagonist has been described so far (BIBP3226; Rudolf et al., 1994). Studies are in progress to find highly selective agonists and antagonists for the various Y_n-R. The transduction systems associated with these receptors are not yet well established because there are reported differences according to the target organs (Aakerlund et al., 1990; Michel et al., 1998). Nevertheless, it appears that all Y_n-R are coupled to G_i and associated with a more or less strong inhibition of cAMP accumulation. Additional signaling responses that are restricted to certain cell types include mobilization of Ca^{2+} from intracellular stores sometimes involving IP_3 and/or inhibition of the Ca^{2+} channel (Perney and Miller 1989; Aakerlund et al., 1990; Selbie et al., 1995).

NPY is present in high concentrations in the mammalian pineal gland (see Mikkelsen and Møller, 1999 for review). Concentrations between 430 and 788 pmol/g have been measured in the rat pineal gland (Chronwall et al., 1985; Møller, 1994). NPY is mainly localized in pineal fibers (except for the little brown rat—(Laemle and Cotter, 1992) and the Syrian hamster—(Schröder, 1986) whose pineal gland contains some NPY-IR cells). A dense NPYergic innervation has been observed in the pineal gland of numerous species, namely the rat (Schon et al., 1985), guinea pig (Schröder and Vollrath, 1986), Syrian hamster (Schröder, 1986), gerbil (Shiotani et al., 1986), sheep (Williams et al., 1989; Cozzi et al., 1992), mink (the only species with rather low NPYergic innervation: Møller et al., 1990b), monkey (Mikkelsen and Mick, 1992), cow (Phansuwan-Pujito et al., 1993), cat (Møller et al., 1994), cotton rat (Matsushima et al., 1994), pig (Kaleczyc et al., 1994), Siberian hamster (Reuss and Olcese, 1995), and European hamster (Møller et al., 1998). The NPY fibers enter the pineal gland mainly through the distal part and end in the perivascular spaces and between the pinealocytes throughout the pineal gland. NPY is partly of sympathetic origin, colocalized with NE, since a large portion of the NPY fibers disappears after SCGx. This has been reported in the rat (Zhang et al., 1991), sheep (Cozzi et al., 1992), cat (Møller et al., 1994), and European hamster (Møller et al., 1998). In the mink, the majority of NPY fibers are of extra-sympathetic origin (Møller et al., 1990b). It is suggested that the extra-sympathetic NPYergic innervation could be of central origin, in particular from the IGL that contains NPY neurons (Card and Moore, 1989), and has a direct neural connection with the proximal part of the pineal gland (Korf and Møller, 1985; Mikkelsen and Møller, 1990; Mikkelsen et al., 1991). It is also possible that NPY could originate from the peripheral ganglia (Møller et al., 1996).

In the rat pineal gland, as in other structures, NPY acts both pre and postsynaptically (Simonneaux et al., 1994a,b). NPY (EC_{50} = 50 nM) inhibits by 45% the presynaptic release of NE induced by high K^+ depolarization. This inhibition is sensitive to pertussis toxin, and independent of, but additive to, α_2-AR inhibition of NE release (Simonneaux et al., 1994b). The Y_2-R agonist NPY (13–36), but not the Y_1-R agonist (Leu^{31}, Pro^{34})-NPY induces a similar inhibition of NE release, suggesting that presynaptic inhibition occurs via activation of the presynaptic Y_2-R, known to be associated with inhibition of AC and sensitive to pertussis toxin (Wahlestedt et al., 1986). It has been reported in other tissues that presynaptic inhibition of NE release via Y_2-R results from complex Ca^{2+}-dependent mechanisms (McCullough and Westfall, 1996; Oellerich et al., 1994). It is noteworthy that NPY and NE, colocalized in the same terminals, can be released differentially, with high-frequency stimulation inducing the release of both NE and NPY and low-frequency stimulation inducing the release of NE only (Torres et al., 1992; May et al., 1995). Therefore, both sympathetic neurotransmitters may have differential effects on pineal activity depending on the intensity of the sympathetic stimulation.

Postsynaptically, NPY acts on two transduction systems. To a small extent it inhibits (20 to 30%; EC_{50} = 5 nM) the increase in cAMP induced by β_1-AR stimulation (Olcese, 1991; Harada et al., 1992; Simonneaux et al., 1994b; Rekasi et al., 1998). It also increases the concentrations of $Ca^{2+}{}_i$, probably via Ca^{2+} influx (Simonneaux et al., 1999). NPY receptors have been characterized pharmacologically (K_D = 1 nM and B_{max} = 40 fmol/mg protein, Olcese, 1991). The rat pineal gland expresses the gene coding for Y_1-R, but not for Y_2-, Y_4-, or Y_5-R (Simonneaux et al., 1994b; Mikkelsen et al., 1999). Inhibition of cAMP is better reproduced by the Y_1-R agonist, [Leu^{31}, Pro^{34}]-NPY, than by the Y_2-R agonist, NPY(13–36) (Simonneaux et al., 1994b). The NPY-induced Ca^{2+} increase in rat pinealocytes is inhibited in the presence of the Y_1-R antagonist, BIBP3226 (Simonneaux and Ribelayga, 2002). These data demonstrate that both postsynaptic effects of NPY are mediated by the Y_1-R. The opposite effects of NPY on cAMP and $Ca^{2+}{}_i$ may explain its complex effects on the MEL synthesis pathway. In vitro studies have shown that NPY stimulates the secretion of 5-HT by 20 to 40%, probably via Ca^{2+}-dependent activation of TPOH activity (Simonneaux et al., 1997c); inhibits to a small extent β_1-AR stimulation of AA-NAT activity (20 to 30%) (Simonneaux and Ribelayga, 2002); and increases by 30 to 50% HIOMT activity, probably via a Ca^{2+}-dependent mechanism (Ribelayga et al., 1997). The effect of NPY on MEL synthesis in vitro is not clearly established. In the rat,

some studies have shown that NPY stimulates basal MEL release and potentiates NE-induced MEL synthesis (Vacas et al., 1987; Mess et al., 1991; Simonneaux et al., 1994b), while other studies reported a moderate (Rekasi et al., 1998; Pfeffer at al., 1999) or powerful (Olcese, 1991) inhibition of NE-induced MEL release. In the sheep, NPY displays no effect on MEL release (Williams et al., 1989). These contradictory results point out the limitations of in vitro experiments in the search for a physiological role of such a neurotransmitter with complex pre and postsynaptic effects. Indeed, an early in vivo study showed that intra-arterially injected NPY stimulated HIOMT activity during the day, and inhibited AA-NAT activity during the night (Reuss and Schröder, 1987).

Although the effect of NPY in vivo on the synthesis of MEL remains to be firmly established, the presence of a dense NPYergic innervation of the pineal gland in numerous mammals, the characterization of specific receptors in the rat pineal gland, and the in vitro observation of cellular and molecular effects of NPY on pinealocytes are strong indicators of an important physiological role of this peptide in the regulation of pineal metabolic activity (Fig. 10A). Daily and circadian rhythms in NPY concentrations have been observed in the rat pineal gland (Shinohara and Inouye, 1994) with a maximal concentration during the first part of the night (ZT 16) and a minimum concentration at the end of the night/beginning of the light (ZT 0). This observation suggests that NPY participates in the expression of the daily rhythm in MEL production. Since NPY moderately stimulates (20 to 50%) HIOMT activity in vitro (Ribelayga et al., 1997) and in vivo (Reuss and Schröder, 1987) and the activity of this enzyme is slightly (30 to 50%) increased at night by cAMP-independent mechanisms (Ribelayga et al., 1997), we suggest that NPY might be the endogenous nocturnal stimulator of HIOMT activity in the rat pineal gland. However, NPY content displays marked seasonal variations in the pineal gland of certain rodent species, for example the European hamster (Møller et al., 1998). This species is of particular interest since it shows large seasonal variations in the length and am-

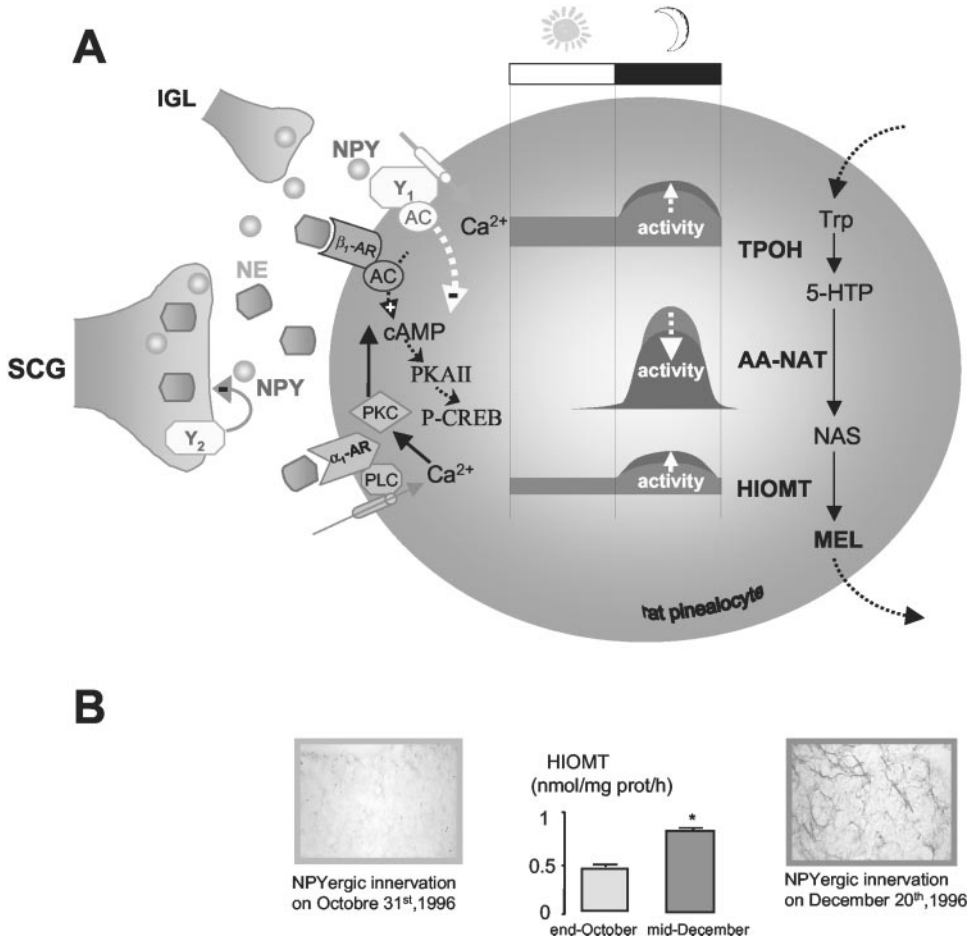

FIG. 10. A, Pre and postsynaptic effects of NPY on the noradrenergic regulation of MEL synthesis in the rat pineal gland. NPY (mainly originating from the SCG and IGL) binds to postsynaptic Y_1-R. On the one hand, NPY inhibits AC activity and therefore reduces the β_1-AR-induced increase in cAMP levels and AA-NAT activity; on the other hand, it increases intracellular levels of Ca^{2+}, which may lead to a moderate increase in TPOH and HIOMT activities. In addition, NPY binds to presynaptic Y_2-R to reduce the release of NE from the sympathetic fibers. B, the seasonal increase in the density of NPY innervation is positively correlated to an increase in HIOMT activity in the pineal gland of the European hamster. The pineal gland of European hamsters sacrificed in October or December were stained for NPY-IR or assayed for HIOMT activity. The increase in NPY-IR observed in December was associated with an increase in HIOMT activity (from Møller et al., 1998, with permission; Ribelayga et al., 1999, with permission).

plitude of the nocturnal MEL peak and the diurnal 5-ML peak (increase from September to December, then decrease until a minimum reached in May/June; Vivien-Roels et al., 1997). The density of the NPYergic fibers, essentially originating from the SCG, increases rapidly from the end of October until mid-December, then returns gradually to minimal values in April (Møller et al., 1998; Fig. 10B). This augmentation is specific for NPY, since during the same period TH activity remained constant (Møller et al., 1998). Interestingly, HIOMT activity is significantly enhanced by 80% from the end of October to mid-December, in association with the increased NPYergic innervation (Ribelayga et al., 1998c; Fig. 10B). Furthermore, these increases are also associated with an augmentation of the peak amplitude of 5-ML (Ribelayga et al., 1998c) and MEL (Vivien-Roels et al., 1997). These results suggest that, in the European hamster, NPY is partly (since the amplitude of the nocturnal MEL peak begins to increase before the increased NPYergic innervation) involved in the seasonal regulation of nocturnal MEL and diurnal 5-ML synthesis via stimulation of HIOMT activity. These experimental data are significant because they indicate for the first time that a neuropeptide may be involved in the annual regulation of the metabolic activity of the pineal gland. In the Siberian hamster, we have also reported photoperiodic regulation of HIOMT activity, which is positively associated with photoperiodic regulation of the amplitude of the nocturnal MEL peak (Ribelayga et al., 2000). We are currently investigating a possible correlation of this with NPY.

It remains necessary to determine the role of NPY pre and postsynaptically in the pineal gland. The in vivo study of Reuss and Schröder (1987) reported that NPY injected at night inhibits rat pineal AA-NAT activity. This nocturnal inhibition could result from presynaptic inhibition of NE release and/or postsynaptic inhibition of the cAMP/AA-NAT/MEL pathway. It is possible that NPY is involved in the rapid inhibition of NE release induced by acute light exposure at night (Drijfhout et al., 1996c). In support of this, various lesion experiments suggest involvement of NPY in the light-induced inhibition of MEL synthesis and release (Dafny, 1980; Cipolla-Neto et al., 1995; Bartol et al., 1997; see *Section V.A.6*). It is noteworthy that NPY may act on MEL synthesis at a presynaptic level on sympathetic fibers, at a postsynaptic level on pinealocytes, and on the blood vasculature of the pineal gland since it displays a powerful vasoconstrictor effect in many tissues, including the pineal gland (Nilsson, 1991).

All of the above in vivo and in vitro experiments point to complex effects of NPY in the daily and seasonal regulation of MEL secretion (see Simonneaux and Ribelayga, 2002 for review). To establish the precise physiological role of NPY in the pineal gland at different times of the daily and annual cycles, it will be necessary to adopt a more direct in vivo approach (e.g., pineal microdialysis to measure the extracellular release of NPY and to test local application of specific NPY ligands on endogenous MEL release; use of antisense molecules for NPY or NPY-R).

3. Vasopressin and Oxytocin. VP and OT were the first peptides isolated from nervous tissue, namely the neurohypophysis (Du Vigneaud et al., 1954). These two peptides are very similar and stem from a common ancestral peptide (see Mohr and Richter, 1994 for review). They are made of nine amino acids with a disulfide bond between the Cys^1 and Cys^6. The sequence of the gene coding for their precursors is very similar and contains a signal peptide, the peptide, and neurophysin (I for OT and II for VP; the carrier of the corresponding peptide). Each gene codes for only one transcript. The mRNA coding for VP and OT, however, can be modified in the 3′ end by a polyadenylated tail that is thought to stabilize the mRNA and/or improve the efficiency of the translation (see Mohr et al., 1992; Gainer and Wray, 1994; Mohr and Richter, 1994 for reviews).

VP and OT were first considered as neurohormones, synthesized in the magnocellular neurons of the hypothalamic supraoptic nuclei (SON) and PVN, transported through the neurohypophysis via hypothalamo-pituitary axons and released in the bloodstream to act on their peripheral target organs (see Argiolas and Gessa, 1991; Richard et al., 1991 for reviews). OT acts essentially on the smooth muscular fibers of the uterus to induce uterine contractions during delivery, and on the myoepithelial cells of mammary glands to induce milk ejection. VP acts primarily on the epithelial cells of the distal kidney tubule to regulate membrane water channel aquaporin to ensure water homeostasis. Additionally, VP induces vasoconstriction and stimulates glycogenesis. VP and OT have also been described as neurotransmitters of the central nervous system (Buijs et al., 1978). These neuropeptides are synthesized in the SON and PVN neurons and in other neural structures (essentially the SCN (VP only) and the bed nucleus of the *stria terminalis*). VPergic and OTergic neurons project to many brain regions, especially the amygdala, lateral septum, hippocampus, cortex, and spinal cord (Buijs et al., 1978, 1988), indicating that they are involved in the regulation of several central functions. OT is involved in learning and memory processes, maternal and sexual behaviors, steroidogenesis, tolerance and dependence mechanisms, and the regulation of the secretion of pituitary hormones (including prolactin). VP is involved in memory acquisition and retention, and in the release of pituitary hormones (see Richard et al., 1991; Mohr et al., 1992; Gainer and Wray, 1994; Mohr and Richter, 1994 for review). VPergic innervation displays gender and seasonal variations, and is dependent on sex hormone concentrations in some brain areas, such that the increase in VP is correlated to an increase in testosterone concentration (see De Vries et al., 1984, 1986, 1994; Hermes et al., 1990; Pévet et al., 1987, for review). Therefore, VP

is involved in the regulation of some seasonal functions such as hibernation (Hermes et al., 1989) and daily torpor (Ouarour et al., 1995). OT and VP are also involved in the transmission of circadian information within the photoneuroendocrine system. In particular, VP, whose promoter gene contains a clock protein-regulated E-box (Jin et al., 1999), is considered to be one of the main SCN outputs involved in the circadian regulation of hormone release (see Buijs and Kalsbeek, 2001 for review).

To date four types of receptors for OT and VP have been characterized. The OT receptor (OT-R) is present in various brain areas, which include the olfactory system, hippocampus, and several hypothalamic nuclei (Freund-Mercier et al., 1987; Dubois-Dauphin et al., 1992; Kermarik et al., 1995). The gene coding for this receptor has been cloned in humans (Kimura et al., 1992) and the rat (Rozen et al., 1995). The hepatic/vascular type of VP receptor (V_{1a}-R) is present in the liver, SCG, vascular system of the central nervous system, and in several brain areas, especially the olfactory bulb, cortex, lateral septum, hippocampus, and a number of hypothalamic nuclei including the SCN and the arcuate nucleus (Tribollet et al., 1988; Dubois-Dauphin et al., 1990, 1992; Theler et al., 1993; Kermarik et al., 1995). The gene coding for this receptor has been cloned in the rat (Morel et al., 1992) and humans (Thibonnier et al., 1994). The pituitary-type VP receptor (V_{1b}-R) is mainly present in the pituitary, but also in other peripheral (intestine, heart) and central (hypothalamus) structures. The coding gene has been cloned in humans (Sugimoto et al., 1994) and the rat (Lolait et al., 1995). The V_2-R is present in the kidney and its coding gene has been cloned in rat (Lolait et al., 1992) and humans (Barberis et al., 1993). OT-R, V_{1a}-R, and V_{1b}-R are all coupled via G_q proteins to PLC to induce PI turnover and $Ca^{2+}{}_i$ increase, whereas the V_2-R is positively coupled to AC (see Birnbaumer, 2000; Gimpl and Fahrenholz, 2001; for reviews). In addition, it has been reported that the V_{1a}-R activates not only PLC, but also phospholipases A and D (Thibonnier, 1992; Briley et al., 1994).

The presence of VP, OT, and some of their metabolites have been reported in the pineal gland of several mammals (Dogterom et al., 1980; Pévet et al., 1980c; Fisher and Fernstrom, 1981; Geelen et al., 1981; Liu et al., 1988; Noteborn et al., 1988). The first studies using radioimmunoassay and high-performance liquid chromatography suggested that these peptides were specific to the pineal gland, giving the gland its antigonadotropic and milk-ejection properties (see Pévet, 1983b; Vaughan, 1984 for review). From 1980, the use of immunocytochemistry demonstrated that VP and OT are usually localized in the pineal fiber endings and not in the pineal cells (Buijs and Pévet, 1980). VPergic and OTergic fibers were observed in the pineal gland of the rat (Buijs and Pévet, 1980), hedgehog (Nürnberger and Korf, 1981), dog (Matsuura et al., 1983), monkey (Ronnekleiv, 1988), cow (Olcese et al., 1993; Badiu et al., 1999; 2001), and pig (Przybylska-Gornowicz et al., 2002). These peptides are thought to originate from the PVN (Buijs and Pévet, 1980; Nürnberger and Korf, 1981). This is strengthened by the demonstration of a monosynaptic connection between the PVN and the pineal gland passing through the *stria medullaris*, using retrograde and anterograde tracing (Korf and Wagner, 1980; Guérillot et al., 1982; Møller and Korf, 1983a,b; Reuss and Møller, 1986; Møller et al., 1990a; Larsen et al., 1991) and electrophysiology (Reuss et al., 1985). Nevertheless, this hypothesis remains controversial (Liu et al., 1991). Recently, using more sensitive molecular biology tools (RT-PCR and ISH), it has also been proposed that VP is synthesized in pineal cells. *Vp*mRNA has been observed in the pineal gland of the rat (Lepetit et al., 1993), cow (Olcese et al., 1993; Badiu et al., 1999), and sheep (Matthews et al., 1993). Several hypotheses can explain that, in contrast, VP-IR cells are absent in the pineal gland: the quantity of synthesized VP is too low to be detected by immunocytochemistry; the *Vp*mRNA is not translated into a peptide, and the detected mRNA is not present in cells but in the nerve endings, as observed in the pituitary (Mohr and Richter, 1993). In contrast to VP, recent data using ISH and immunohistochemistry have demonstrated the presence of a few neuron-like cells synthesizing and containing OT in the bovine pineal gland (Badiu et al., 2001). In general, the content of VP and OT in the rat pineal gland is rather low (20 (VP) and 14 (OT) fmol/pineal; Liu and Burbach, 1987).

In an early experiment with perifused rat pineal glands we reported that high doses of VP and OT potentiate (by 1.5- to 2.5-fold) the β_1-AR-induced stimulation of MEL synthesis (Simonneaux et al., 1990b). However, in a more sensitive model using cultured pineal cells, we found that at physiological doses (ED_{50} = 7 nM) only VP could potentiate the β_1-AR-induced synthesis of MEL (Simonneaux et al., 1996a). VP potentiation of MEL synthesis occurs for low and moderate, but not high, β_1-AR stimulation. The VP effect occurs via potentiation of cAMP accumulation (Simonneaux et al., 1996a) and consequent AA-NAT activation (Stehle et al., 1991). The observation that VP potentiation of MEL synthesis is inhibited by a V_{1a}-R antagonist (Simonneaux et al., 1996a) suggested the presence of V_{1a}-R receptors in the rat pineal gland. By using a specific linear antagonist of V_{1a}-R (Barberis et al., 1995) we have shown that membranes isolated from the rat pineal gland possess a low density (13 fmol/mg protein) of high affinity V_{1a}-R (K_D = 10 pM). However, because the pineal gland contains numerous blood vessels and the V_{1a}-R is highly expressed in endothelial cells, we further characterized the localization of these receptors in the rat pineal gland. The combination of binding studies using an iodinated V_{1a}-R ligand together with 5-HT immunohistology on dissociated pineal cells showed that a small portion (20

to 30%) of isolated pinealocytes is indeed endowed with V_{1a}-R. In addition, we found that the pharmacological profile of these pineal receptors is similar to that of the V_{1a}-R and that the gene coding for V_{1a}-R but not for the other VP/OT receptors is expressed in the cultured pinealocytes (Simonneaux, unpublished data). All these observations are in good agreement with the presence of V_{1a}-R in rat pinealocytes and around blood vessels, as suggested by previous studies (van Leeuwen et al., 1987; Ostrowski et al., 1994; Tribollet et al., 1999). In several central structures activation of V_{1a}-R induces an IP_3-dependent increase of $Ca^{2+}{}_i$. The effect of VP on the intracellular levels of Ca^{2+} in dissociated pineal cells was therefore assessed (Simonneaux, unpublished data). We observed that numerous pineal cells respond to VP by a transient increase in $Ca^{2+}{}_i$, an effect abolished by a V_{1a}-R antagonist. Among these cells were a number of pinealocytes and fibroblast-like cells. These data are in agreement with the observation that VP increases PI turnover in the rat pineal gland (Novotna et al., 1995) but not with that of Schomerus et al. (1995) who reported no effect of VP on the $Ca^{2+}{}_i$ level in cultured rat pinealocytes.

The presence of VPergic nerve endings in the pineal perivascular space (Buijs and Pévet, 1980) and the location of a high density of V_{1a}-R in the pineal blood vessels (Ostrowski et al., 1994; Simonneaux, unpublished observations), which are associated with the well characterized vasoconstrictor effect of VP, suggest that VP could also modulate blood flow in the pineal gland.

Interspecific differences in the effect of VP and OT are possible. Whereas VP is stimulatory in the rat pineal gland, it appears to be inhibitory in the bovine pineal gland (Olcese et al., 1993). In contrast to the rat, OT may be a transmitter in the sheep pineal gland since OT-R, but not V_{1a}-R, have been identified (Rahmani et al., 1997). To date, however, only the absence of a VP or OT effect on basal cAMP levels has been reported in cultured sheep pineal glands (Morgan et al., 1988).

In the rat pineal gland the above in vitro studies showed that VP, probably originating from the hypothalamic PVN, binds to specific V_{1a}-R in a subpopulation of pinealocytes, stimulates PI turnover, and increases $Ca^{2+}{}_i$ levels to potentiate the NE/β_1-AR/cAMP/AA-NAT/MEL pathway (Fig. 11A). It remains necessary to delineate the precise physiological role of VP on MEL synthesis. Recently, we have developed the in vivo technique of pineal microdialysis to study the effect of locally infused drugs on *endogenous* MEL secretion from the rat pineal gland (Barassin et al., 1999). By using this in vivo approach it has been shown that VP infused into the pineal gland at the beginning of the MEL rise (but not when the

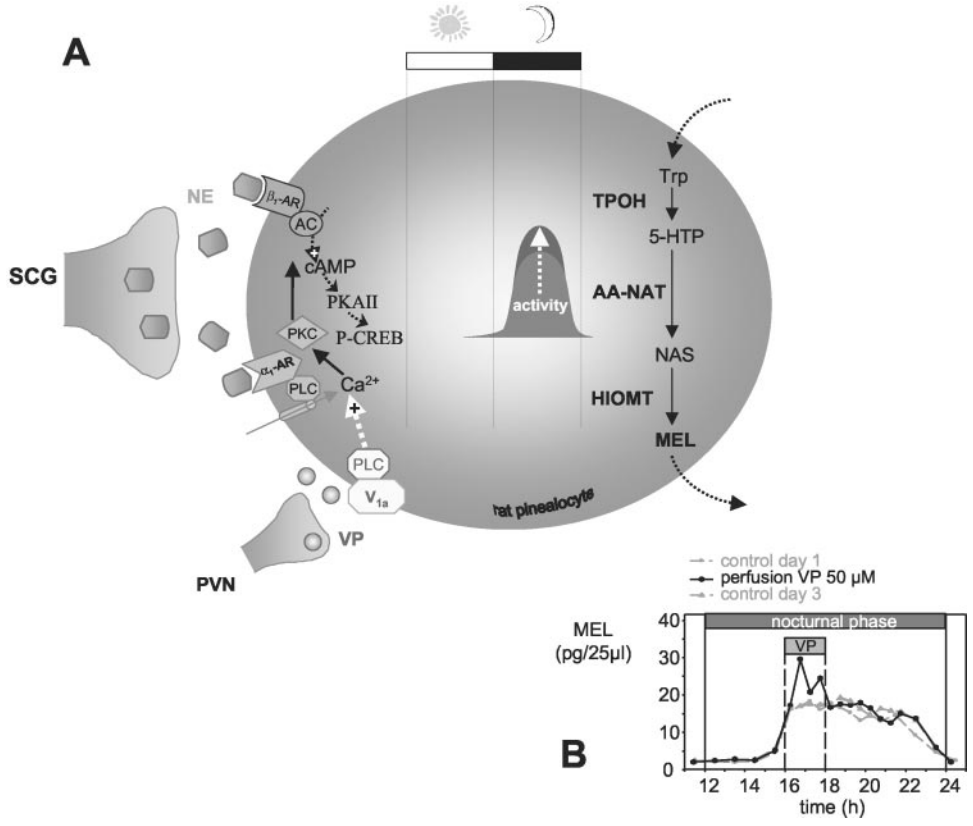

FIG. 11. A, intracellular effects of VP on the MEL synthesis pathway in rat pinealocytes. VP, originating from the PVN, binds to PLC-coupled V_{1a} receptor, increases the intracellular level of Ca^{2+}, and potentiates the β_1-AR-induced increase in AA-NAT activity and MEL synthesis and release. B, in vivo infusion of VP potentiates the endogenous nocturnal release of MEL in the rat pineal gland. Endogenous release of MEL was measured by intrapineal microdialysis for three consecutive nights in the pineal gland of one rat. On the second day of the experiment infusion of 50 μM VP induced a significant increase in the endogenous release of MEL lasting for the duration of the VP infusion (from Barassin et al., 2000, with permission).

release is maximal) further increases the endogenous MEL secretion (Fig. 11B; Barassin et al., 2000). This in vivo observation is thus in good agreement with our previous in vitro observations and strongly indicates that VP is able to modify MEL synthesis and release. Whether this VP-induced modification occurs on a daily and/or a seasonal basis remains to be determined. Some studies report a small daily variation in VP and OT content in the rat pineal gland, with nocturnal values being slightly (28%) higher (Gauquelin et al., 1988; Liu and Burbach, 1988). These variations persist in D/D and could result from a nocturnal decrease in aminopeptidase activity (Liu and Burbach, 1988). A lesion of the *stria medullaris*, from where the PVN neurons forward their fibers to the pineal gland, significantly decreased AA-NAT (−50%) and HIOMT (−35%) activity measured 4 h after the beginning of the night (Møller et al., 1987). This lesion also produced a significant reduction of the amplitude of the nocturnal MEL peak (Reuss et al., 1987). These in vivo observations suggest that VP, originating from the hypothalamic PVN, potentiates the nocturnal NAergic stimulation of MEL synthesis. In addition, pineal VP and OT concentrations display a large, temporary increase from July to mid-August (14 to 82 fmol VP/pineal and 20 to 193 fmol OT/pineal) in rats kept in a constant photoperiod (Liu and Burbach, 1987; Liu et al., 1991). This summer peak in peptide concentrations is preserved after SCGx, indicating that NAergic stimulation is not responsible for this increase (Prechel et al., 1989). It has been suggested that the summer increase in VP and OT originates in the pineal gland itself since a simultaneous increase has not been observed in the SCN and PVN (Liu et al., 1991). No seasonal variation in *Vp* or *Ot* mRNA expression was observed, however, using RT-PCR on rat pineal cDNA using specific primers for *Vp* and *Ot* mRNA (Simonneaux, unpublished observations). Seasonal variations in the pineal content of VP and OT have also been described in hedgehog (Nürnberger and Korf, 1981), the VPergic and OTergic innervation of the pineal gland being very low in summer and increasing in winter. A marked seasonal variation in OT content has also been observed in the bovine pineal gland, with a 3-fold higher value in September compared to the other months (Badiu et al., 2001). In addition, the quantity of VP increases in the pineal gland of female rats at the end of proestrus/beginning of estrus (Moujir et al., 1990b). It is possible that this increase originates from the bloodstream since VP, whose synthesis depends on sex hormones (De Vries et al., 1986), reaches its highest level during proestrus and increases after estradiol administration (Skowsky et al., 1979). The observation of seasonal variations in pineal VP and OT suggests that these neuropeptides are involved in the seasonal regulation of pineal metabolic activity, but this hypothesis requires further study.

4. Somatostatin. SOM has been isolated from sheep hypothalamic extracts and identified as being an inhibitor of growth hormone release. SOM was first named after this effect on growth hormone (somatotropin releasing inhibitor factor; Brazeau et al., 1973). It is a cyclic tetradecapeptide with a disulfide bridge between the Cys^3 and Cys^{14}, also existing under a longer form of 28 amino acids. SOM is widely distributed in the central nervous system and in peripheral organs where it is involved in neuroendocrine, motor, and cognitive functions. SOM also regulates the differentiation and proliferation of normal and tumor cells (see Rubinow et al., 1995; Schindler et al., 1996, for review).

In 1992/1993, five receptors for SOM (sst1–5) were characterized and found to be located in many peripheral and central areas. Whereas the *sst1*, *sst3–5* genes each generate a single receptor protein, alternative splicing of *sst2* mRNA gives rise to two isoforms, *sst2A* and *sst2B* (Yamada et al., 1992a,b, 1993; Xu et al., 1993; Csaba and Dournaud, 2001). The pharmacology of these receptors is not well known because of the lack of specific agonists and antagonists. Two groups have been defined: SST1 (*sst2, sst3, sst5*) with a high affinity for the short SOM analogs (especially octreotide) and SST2 (*sst1* and *sst5*). The transduction signaling pathway associated with these receptors is not clearly established. Studies performed on recombinant receptors expressed in a cell line have produced various intracellular effects. The five receptors are generally associated with an inhibition of AC, but also activation of PLC and type A_2 phospholipase, modulation of the Na^+/H^+ pump, modulation of Ca^{2+} and K^+ fluxes, and activation of MAP kinases (see Raulf et al., 1996; Schindler et al., 1996; Csaba and Dournaud, 2001; for review).

The presence of SOM in the pineal gland was shown for the first time by Pelletier et al. (1975) in the rat (and later on by Pévet et al., 1980b; Finley et al., 1981; Webb et al., 1984; Møller et al., 1995) then in the hamster, gerbil, mouse (Webb et al., 1984), sheep, pig (Lew and Lawson-Willey, 1987), cow (Peinado et al., 1989; Møller et al., 1992), and human (Bouras et al., 1987). The rat pineal gland contains approximately 0.3 to 3 ng SOM/mg protein (Webb et al., 1985). SOM is mainly observed in the nerve fibers and in some neuronal-type cells of the pineal gland in the rat (Møller et al., 1995), European hamster (Møller, personal communication), sheep, cow (Viader et al., 1990, although discussed by Møller et al., 1992), and pig (Przybylska-Gornowicz et al., 2000a). In the rat, SOM is probably synthesized by the pineal cells since they contain mRNA coding for this peptide (Mato et al., 1993, 1997; Møller et al., 1995). The SOMergic fibers are not of sympathetic origin since the pineal content of SOM is not modified after SCGx (Webb et al., 1984, 1985). It is proposed that these fibers could be of central origin because the number of SOMergic fibers and the peptide concentration are 4 times higher in the

proximal than in the distal area of the gland (Peinado et al., 1989; Møller et al., 1992).

The expression of functional SOM receptors in the pineal gland remains to be established. In the rat pineal, mRNA coding for *sst2* was detected by RT-PCR (Mato et al., 1997) but the corresponding receptor could not be found by autoradiography (Sabry and Suzuki, 1993). In the pig pineal gland, immunoreactivity for only one receptor subtype (SST3) was demonstrated (Przybylska-Gornowicz et al., 2000a). In the ovine pineal gland, no *sst1* mRNA could be detected (Debus et al., 2001).

While the presence of SOM in the pineal gland has been established for a long time, its effect on pineal metabolic activity has not yet been determined. Some authors have reported a lack of effect of SOM on basal or stimulated MEL synthesis (Kaneko et al., 1980; Morgan et al., 1988; Simonneaux, unpublished results) and intravenous injections of SOM had no effect on the nocturnal peak of MEL (Webb et al., 1985). Other studies, however, have reported that SOM potentiates the NE-induced synthesis of MEL (Mess et al., 1991) or activates acetyl coenzyme A hydrolase by protein-thiol/disulfide exchange mechanisms (Namboodiri et al., 1982). The synthesis of SOM in some pineal cells suggests that this peptide could display paracrine effects on other pineal functions (for example, on the number of synaptic ribbons: Gupta et al., 1992). It is also proposed that SOM may be involved in pineal development and cell differentiation since the number of SOM-containing cells (Viader et al., 1995) and the quantity of mRNA coding for this peptide (Mato et al., 1997) decrease from 8 to 15 days after birth in the rat pineal gland. Similarly, the number of SOM-containing pineal cells and fibers decrease with age in the pig pineal gland (Przybylska-Gornowicz et al., 2000a). A general role in neurogenesis has been proposed for SOM by studies in the cerebellum (Gonzalez et al., 1992; Laquerrière et al., 1992). In addition, the possibility of a presynaptic effect of SOM on NE release is worthy of study, since the SCG possess SOM receptors (Manthy et al., 1992) and SOM inhibits Ca^{2+} currents of the sympathetic neurons in the rat (Shapiro and Hille, 1993) and NE release by sympathetic neurons in the chicken (Boehm and Huck, 1996).

It is interesting to note that pineal SOM content displays a daily variation with a peak at the end of the day (ZT 13) in several species (Webb et al., 1985, 1988) and a seasonal variation with higher values during autumn/winter (Peinado et al., 1990).

5. Substance P. sP was discovered in 1931 by Von Euler and Gaddum because of its property of decreasing arterial pressure via vasodilatation of the peripheral vascular system. Its 11-amino acid sequence was identified by Chang et al. (1971). sP belongs to the neurokinin/tachykinin family (NK), which consists of NKA and NKB in addition to sP. These NK are coded by two precursor genes: *Ppt (preprotachykinin)-A* coding for sP and NKA, and *Ppt-B* coding for NKB (see Regoli et al., 1994 for review). Each of the three peptides displays an optimal affinity for one of the three NK receptors: NK_1 (sP), NK_2 (NKA), and NK_3 (NKB). These receptors are coupled to an activation of AC and/or PLC. sP is particularly involved in the transmission of nociception. However, it is also involved in other biological functions such as regulation of arterial pressure, secretion of several hormones (pancreatic, pituitary), release of some neurotransmitters (especially ACh, DA), and immune and inflammatory functions (see Snijdelaar et al., 2000 for review). sP is also involved in the transmission of photic information from the retina to the SCN (Mikkelsen and Larsen, 1993; Shirakawa and Moore, 1994) and displays a critical role together with Glu in photic resetting of the circadian clock (Shibata et al., 1992; Challet et al., 1998; Kim et al., 2001).

sP was one of the first neuropeptides identified in the mammalian pineal gland (Ljungdahl et al., 1978). An sPergic innervation was described in the pineal gland of the rat (Ronnekleiv and Kelly, 1984), gerbil (Shiotani et al., 1986), monkey (Ronnekleiv, 1988), cow (Møller et al., 1993), cotton rat (Matsushima et al., 1994), tree shrew (Kado et al., 1999), and pig (Przybylska-Gornowicz et al., 2000b). The sPergic innervation is dense, dispersed through the whole gland, and terminates in the perivascular space and between the pineal cells (see, for example, Ronnekleiv and Kelly, 1984). The possibility that sPergic fibers originate from neurons of the habenular nuclei, at least in the rat and cow, rely on the following observations: 1) some sP-containing neurons of the habenular area project their axons via the habenular commissure toward the proximal part of the pineal gland (Ronnekleiv and Kelly, 1984; Møller et al., 1993); 2) pineal sPergic innervation is not modified after SCGx (Ronnekleiv and Kelly, 1984; Matsuura et al., 1994; Kado et al., 1999); 3) a direct neural connection between the habenular nuclei and the pineal gland was demonstrated by lesion experiments (Ronnekleiv and Møller, 1979; Møller and Korf, 1983b), electrophysiology (Reuss et al., 1984), and tracing studies (Møller and Korf, 1983b). In addition, pineal sP is proposed to originate from the trigeminal ganglia (see Shiotani et al., 1986; Reuss et al., 1992a; Reuss, 1999 for review). NKA, another tachykinin, has been found in the rat pineal gland, its content being increased following castration or SCGx (Debeljuk et al., 1998).

Despite the dense pineal sPergic innervation, no effect of sP on pineal metabolic activity has yet been reported (Yuwiler, 1983a; Govitrapong and Ebadi, 1986; Mess et al., 1991; Simonneaux, unpublished results). However, an NK1 type of sP receptors has been characterized in the bovine pineal gland (Govitrapong and Ebadi, 1986). Additional studies are necessary to establish the role of sP in the mammalian pineal gland. Localization of NK1 receptors on pineal cell types would help to delineate its function. It might also be interesting to study the effect of sP on NE release (sP modulates NAergic transmission

in some tissues, Yusof and Coote, 1987) and on pineal blood flow.

6. Calcitonin Gene-Related Peptide. CGRP is a cyclic peptide (with a disulfide bond between Cys^2 and Cys^7) of 37 amino acids (see Wimalawansa, 1996 for review). It is generated by the alternative splicing of a primary mRNA transcript from the gene coding for calcitonin (CT; Amara et al., 1984). The *CT/CGRP* gene consequently codes for two peptides, CT and/or CGRPα, according to the tissue type (CT in the thyroid, CGRP in the nervous system and various peripheral structures, especially the cardiovascular system). There is another gene that codes only for CGRPβ. CGRP is found in various nervous structures (especially the spinal cord, trigeminal ganglia, pituitary gland) and in the cardiovascular system. CGRP induces strong vasodilatation (Brain et al., 1985) and is involved in the regulation of vascular tonus and the blood flow of various organs. It is also involved in the ascending sensory pathway from the periphery to the central nervous system as well as in the regulation of immune and inflammatory functions, secretion of pituitary hormones, secretion of pancreatic and gastric enzymes, and cell proliferation and growth. CGRP is present in the mouse SCN and IGL neurons, indicating that it could be involved in the mammalian circadian system (Park et al., 1993). Two receptor types have been identified: $CGRP_1$ and $CGRP_2$ are distinguished by their affinity for the antagonist CGRP 8-37 ($CGRP_1$) or the agonist ($CGRP_2$). These receptors display a wide distribution in the central nervous system and the periphery. Activation of these receptors increases the intracellular level of cAMP, but other signal transduction systems may also be involved (Juaneda et al., 2000).

CGRP-containing fibers have been identified in the pineal gland of the gerbil (Shiotani et al., 1986), rat (Reuss et al., 1992a; Matsuura et al., 1994), cotton rat (Matsushima et al., 1994), and tree shrew (Kado et al., 1999). In the cotton rat, CGRP fibers arrive via the conarian nerve; they are largely spread out in the superficial pineal gland, being rare in the stalk and deep pineal gland, and absent in the habenular and posterior commissures (Matsushima et al., 1994). In the rat, CGRP-containing fibers are abundant in the superficial pineal gland but do not disappear after SCGx (Matsuura et al., 1994). Using tracing techniques and immunocytochemistry in the gerbil pineal gland, Shiotani et al. (1986) have shown that CGRP may originate from the trigeminal ganglia (see Reuss, 1999 for review). It is noteworthy that in several species CGRP has been found in SCG neurons; however, it is not established whether these neurons project to the pineal gland (Lee et al., 1985). Despite the dense CGRP fiber innervation of the pineal gland of several species, no effect of this peptide on pineal metabolism has yet been found. Given its strong vasodilator effect, it would be interesting to study the effect of CGRP on the regulation of pineal blood flow.

7. Secretoneurin. SN is a 33-amino acid peptide discovered in the nervous system in 1993 (Kirschmair et al., 1993). It is synthesized from secretogranin II, which belongs to the chromogranin family (Vaudry and Conlon, 1991). These large secretory proteins are located in the large vesicles of various endocrine and nervous tissues (Fischer-Colbrie et al., 1995). In the brain over 90% of secretogranin II is metabolized into SN. SN occurs in high concentrations in the hypothalamus and median eminence, with lower levels in the lateral septum, habenular nuclei, and *locus coeruleus*. SN specifically activates various cell functions including the migration of monocytes, eosinophils, fibroblasts, and smooth muscle cells, which suggests that the peptide may modulate inflammatory reactions (Wiedermann, 2000). In the central nervous system it may modulate neurotransmission since it stimulates DA release in the striatum (Agneter et al., 1995). Secretoneurin G-protein-linked receptors have been functionally characterized (Schneitler et al., 1998). The description of SN colocalized with NE in the SCG neurons (Klimaschewski et al., 1996b) prompted us to study this peptide in the rodent pineal gland (Simonneaux et al., 1997a).

SN and larger intermediate forms were present in the pineal gland of the three rodents studied (rat, Syrian hamster, Siberian hamster) with interspecies differences. SN-IR was higher in the female Syrian hamster (122 fmol/pineal) than in the rat (34 fmol/pineal) and Siberian hamster (undetectable level). In the rat, SN-IR decreased by 50 to 60% in animals maintained in L/L or SCGx, indicating a partial sympathetic origin. A few fibers were present in the proximal part of the gland, apparently coming from the deep pineal gland via the stalk, indicating a partial central origin of the SN fibers as well (possible origin: some parts of the geniculate complex, some hypothalamic areas, the habenula). In the rat pineal gland there were no SN-IR cells. In the Syrian hamster, SN-IR was present not only in fibers but also in several "neuron-like" cells of the pineal gland. In the Siberian hamster pineal gland there were very few SN-IR fibers and cells. They were no gender differences in the rat SN-IR, but in the Syrian hamster SN-IR was significantly higher in females than in males. Preliminary data indicate that this difference could be related to the sex hormones, since castration induced an increase (from 25 to 52 fmol/pineal) of SN-IR in the pineal gland of male Syrian hamsters raised in LP (Simonneaux and Fisher-Colbrie, unpublished results).

In cultured rat pinealocytes we have observed that SN moderately inhibits intracellular concentrations and release of 5-HT. The effect of SN on MEL release was less and may result from the inhibitory effect on 5-HT synthesis. The mechanism and sites of action of SN are still to be determined. In addition, colocalization of SN with NE in the sympathetic fiber endings suggests that the peptide may have a presynaptic effect on NE release.

8. Hypocretin. Recently, two neuropeptides selectively expressed in the hypothalamus have been identified and found to exert neuroexcitatory and food-stimulating activities. They have been termed HCRT (de Lecea et al., 1998) or orexin (Sakurai et al., 1998) 1 and 2. In addition, these peptides are involved in cardiovascular function, hormone homeostasis, and sleep-wake behavior (see Sutcliffe and de Lecea, 2000 for review). The use of HCRT knockout mice has demonstrated a major involvement of HCRT in the pathophysiology of narcolepsy (Chemelli et al., 1999; Siegel, 1999). HCRT-1 (33 amino acids) and HCRT-2 (28 amino acids) bind to orexin receptors. HCRT-1 binds better to orexin-1 than orexin-2 receptors, whereas both peptides bind with a similar affinity to orexin-2 receptors (Sakurai et al., 1998).

Neurons containing HCRT are exclusively located in the area of the lateral hypothalamus and widely project to numerous regions of the central nervous system, such as various hypothalamic nuclei, *locus coeruleus*, septal nuclei, bed nucleus of the *stria terminalis*, various thalamic nuclei, and spinal cord. (Peyron et al., 1998). The recent finding that food intake may affect circadian clock entrainment (Challet et al., 1996) and the occurrence of neural connections between the lateral hypothalamus nuclei and the pineal gland has led us to investigate the possibility of HCRT regulation of pineal metabolism in the rat (Mikkelsen et al., 2001).

The rat pineal gland was found to receive a strong central HCRTergic input, with fibers running via the medial habenular nuclei and the habenular commissure. HCRTergic fibers end mainly in the deep pineal gland, a few of them continuing via the pineal stalk to the proximal part of the superficial gland. The pineal gland was shown to express orexin-2 but not orexin-1 receptors, indicating that HCRT is a putative neurotransmitter involved in the regulation of pineal metabolism. Indeed, HCRT-2 was able to partially inhibit (by about 30%) the ISO-stimulated increase in AA-NAT activity and MEL release in cultured rat pinealocytes (Mikkelsen et al., 2001). These data suggest that HCRT released by central fibers modulates the stimulatory sympathetic input of the pinealocytes. Interestingly, the release of HCRT from the hypothalamic neurons shows a significant day/night variation with higher levels at nighttime, during the active phase in the rat (Yoshida et al., 2001). These findings support an involvement of this hypothalamic peptide in the daily rhythm of MEL synthesis. Additionally, it would be of interest to study whether these food-regulating peptides are also involved in the adaptation of photoperiodic animals to the seasonal changes in food availability.

9. Delta-Sleep Inducing Peptide. DSIP is a 9-amino acid peptide that can promote sleep in animals under certain conditions. In addition, DSIP displays several other physiological effects including modification of thermoregulation, heart rate, blood pressure, and pain threshold, some of these effects being circadian cycle-dependent (see Yehuda and Carasso, 1988 for review).

When injected into the bloodstream, DSIP accumulates in the pineal gland (Graf and Kastin, 1984), a property that led us to study its effect and mechanism of action in the rat pineal gland (Ouichou and Pévet, 1992; Ouichou et al., 1992). Although it was previously shown that in vivo DSIP inhibits AA-NAT activity and MEL production to a small extent at the beginning of the night (Graf et al., 1985; Oaknin et al., 1986), we have observed that in vitro DSIP infusion of perifused rat pineal glands induces a large, rapid, and dose-dependent stimulation of the release of MEL as well as 5-ML and 5-HT. This stimulatory effect is independent of an increase in cAMP levels. The effect of DSIP, however, is abolished in presence of a peptidase inhibitor or the TPOH inhibitor, *p*CPA. In addition, an infusion of Trp on perifused pineal glands displays a similar stimulation of MEL, 5-HT, and 5-ML release. These observations indicate that DSIP stimulates the synthesis and release of the several pineal indoles via a "release" of Trp (first amino acid in the DSIP sequence) generated by proteolysis. The stimulatory effect of DSIP on MEL synthesis (during the night) appears contradictory with its sleep-promoting effect (during the day) in a nocturnal animal. This ambiguity may be explained by its indirect effect that may be delayed in the nighttime. Comparison of DSIP effects in nocturnal and diurnal animals may resolve this question.

10. Natriuretic Peptides. The natriuretic peptide family is composed of three peptides: atrial (ANP), brain (BNP), and C-type (CNP) natriuretic peptides (see Imura et al., 1992 for review). They are associated with a particular signal transduction system inducing the synthesis of cGMP following activation of different membrane receptors containing GC: mainly GC-A (binding preferentially ANP and less BNP) and GC-B (binding CNP). ANP and BNP are mainly secreted by the heart to regulate blood pressure whereas CNP is mainly produced in the brain and in neuroendocrine organs.

In cultured rat pinealocytes ANP, BNP, and CNP were reported to produce an increase in cGMP levels, suggesting the presence of two types of receptors: GC-A and GC-B (Olcese et al., 1994). Further studies in the rat pineal gland, however, demonstrated a high density of GC-B receptors (not GC-A), whose activation induced a large increase in cGMP levels (Müller et al., 2000). These observations, however, were not confirmed (Spessert et al., 1992). The bovine pineal gland also expresses GC-A and GC-B receptors, activation of which also increases the intracellular concentration of cGMP (Middendorff et al., 1996). In addition, a small population of bovine pineal cells contains CNP associated with synaptic vesicles, suggesting an autocrine/paracrine role for this peptide in the pineal gland (Middendorff et al., 1996). These findings indicate that the CNP/GC-B/cGMP pathway may be of importance in pineal physiol-

ogy, although the natriuretic peptides have no effect on the synthesis and release of MEL (Olcese et al., 1994). Pineal cGMP may be involved in the gating of an ion channel (Schaad et al., 1995b) or in the activation of the MAPK pathway (Ho et al., 1999). It is noteworthy also that these peptides are able to modulate NAergic transmission in the hypothalamus, especially by increasing the uptake of NE and reducing spontaneous K^+-induced NE release (Vatta et al., 1996).

11. Angiotensin. As early as 1975, Haulica et al. detected a high activity of renin, one of the enzymes involved in the formation of angiotensin II (Ang II) from angiotensinogen, in the mammalian pineal gland. Later, Baltatu et al. (1998, 2002) reported that a local renin-angiotensin system is present and functional in the rat pineal gland. Angiotensinogen mRNA is localized in the pineal astrocytes whereas the angiotensin receptor type A_{1b} is expressed in the pinealocytes, suggesting a paracrine function of angiotensin within the pineal gland (Baltatu et al., 1997, 1998). Both in vivo and in vitro studies showed that the A_{1b} receptor antagonist losartan significantly reduces the synthesis of most pineal indoles, in particular 5-HTP, 5-HT, and MEL, independently of AA-NAT activity and probably in parallel with a reduction in TPOH activity (Baltatu et al., 2002). These observations are in agreement with earlier studies reporting a stimulatory effect of Ang II on 5-HT synthesis (Haulica et al., 1980), on NE release, and hydroxy and methoxyindole production (Finocchiaro et al., 1990). Similarly, the regulation of melatonin synthesis is also altered in transgenic rats either carrying an additional mouse renin gene (Enzminger et al., 2001) or with inhibited production of angiotensinogen (Baltatu et al., 2002). These studies suggest that the pineal gland has a local renin-angiotensin system with Ang II, synthesized by the astrocytes, exerting a tonic activation of TPOH activity. In addition, angiotensin-converting enzyme in the pineal gland is under negative control by NE released from the pineal sympathetic nerves (Nahmod et al., 1982).

12. Opiate Peptides. Opiates stem from three precursor families: proopiomelanocortin (proopiomelanocortin, giving the β-endorphins; MSH; and corticotrophin), pro-Enk (giving Leu-Enk and Met-Enk), and prodynorphins (giving the dynorphins A and B, and neoendorphins).

The pineal gland of several species contains fibers and cells IR to various opiates. Fibers containing some opiates, especially Leu-Enk and Met-Enk, β-endorphins, and dynorphin, have been observed in the pineal gland of the guinea pig (Schröder et al., 1988), human (Moore and Sibony, 1988), cow (Cherdchu et al., 1989; Møller et al., 1991a), European hamster (Coto-Montes et al., 1994), and tree shrew (Phansuwan-Pujito et al., 1998). The origin of these fibers is not known but could be the SCG, habenular nuclei, trigeminal ganglia, or parasympathetic ganglia (Schröder et al., 1988). In several species, namely the guinea pig (Schröder et al., 1988), rat (Aloyo, 1991), and European hamster (Coto-Montes et al., 1994), cells of the pineal gland have been shown to synthesize opiates, especially Enk. In the bovine pineal gland most of opiate receptors are of the δ-type and fewer of the μ subtype (Aloyo, 1992; Govitrapong et al., 1992, 2002; Aloyo and Pazdalski, 1995). In mice, high levels of mRNA coding for δ opiate receptors have been observed in the pineal and pituitary gland (Bzdega et al., 1993). In contrast, only low levels of δ and μ opiate receptor mRNA expression were found by RT-PCR in the rat pineal gland (Chetsawang et al., 1999).

Most endorphins and Enk display a stimulatory effect on MEL synthesis in vivo (Lissoni et al., 1986; Esposti et al., 1988; Stankov et al., 1990a) and ex vivo (MEL: Stankov et al., 1990a; AA-NAT: Govitrapong et al., 1992). It has been proposed, however, that this opiate-induced stimulation occurs via NAergic transmission (Fraschini et al., 1989; Stankov et al., 1990a). In support of this several in vitro studies were unable to show any stimulatory effect of Met-Enk, Leu-Enk, or β-endorphin on MEL synthesis (Kaneko et al., 1980; Simonneaux, unpublished results). One study, however, has demonstrated a positive effect of high concentrations of morphine (>50 μM) on AA-NAT activity and MEL production in the bovine pineal gland (Govitrapong et al., 1992, 1998).

The opiates are also considered to be the endogenous ligands for the σ receptors. These receptors were characterized in the rat (with a high density: Jansen et al., 1990) and sheep (Abreu and Sugden, 1990) pineal gland. Two studies have shown contradictory results on the effect of activation of these receptors on MEL synthesis. One study has reported that the DA/σ nonselective agonist haloperidol inhibits NE-induced MEL release via inhibition of cAMP production and PI turnover (Olcese, 1995). The other study, in contrast, has reported a stimulatory effect of a σ1 ligand on ISO-induced daytime MEL production and on endogenous nighttime MEL synthesis (Steardo et al., 1996).

An association between MEL/opiates/analgesia, especially the possibility that nocturnal endogenous MEL has analgesic and hypnotic properties, has been discussed extensively by Fraschini et al. (1989) and Ebadi et al. (1998).

αMSH is a 13-amino acid peptide thought to play a special role in the mammalian pineal gland. High concentrations of αMSH (180 pg/gland in the rat) have been found in the pinealocytes of several species (Oliver and Porter, 1978; Vaudry et al., 1978; Pévet et al., 1980b; Schröder et al., 1988) suggesting an auto/paracrine role of this peptide in the pineal gland (Pévet et al., 1980b). However, its role in the regulation of pineal metabolism has not been clearly established. αMSH decreased the NAergic stimulation of cAMP production in the rat pineal gland (Sakai et al., 1976). In the Siberian hamster an intraperitoneal injection of 200 ng αMSH induced a decrease in 5-HT concentrations and AA-NAT activity,

while higher concentrations (20 μg) decreased MEL secretion without modification of AA-NAT activity (Oaknin et al., 1987). The role of this peptide would be interesting to re-examine as it exhibits a day/night rhythm with higher values peaking at the end of the night/beginning of the day (ZT 1) that persists in D/D or after SCGx (O'Donohue et al., 1980).

13. Luteinizing Hormone-Releasing Hormone. In the historical context of the search for an anti or progonadotropic role of the pineal gland, the effects of peptides of the hypothalamo-pituitary system have been studied in the mammalian pineal gland (see White et al., 1974; Pévet, 1981; for review; Noteborn et al., 1992; Park et al., 1995). It is probable that some of these peptidergic hormones are transported by the blood. Some of these peptides, especially LHRH (Redding and Schally, 1973), radioactively labeled and injected into the bloodstream, accumulate in the pineal gland. In addition, the presence of LHRH-IR fibers was reported in the pineal gland of the rat (Piekut and Knigge, 1981), dog (Matsuura et al., 1983), and monkey (Ronnekleiv, 1988). In the dog these fibers enter the pineal gland via the posterior and habenular commissures. LHRH-IR neurons have been observed in the habenular commissure and may send fibers toward the pineal gland (Barry, 1979). Finally, it has been proposed that some pineal cells synthesize LHRH or a LHRH-like peptide (Pévet et al., 1980b). Until now, few studies have reported an effect of this peptide on pineal metabolism: it is proposed to stimulate HIOMT activity (Cardinali et al., 1976; Cardinali and Vacas, 1979), regulate the formation of granular vesicles and the process of protein and/or peptide secretion (Haldar-Misra and Pévet, 1983), and increase MEL secretion, although only moderately (Mess et al., 1991), mainly via an activation of AA-NAT (Hosaka et al., 2002). Seasonal variations of LHRH have been observed in the pineal gland of the rat (with a maximum in March/May; Joseph, 1976) and sheep (King and Millar, 1981).

14. Peptides to Come. In the expanding field of peptide research, new peptides that regulate/modulate several functions are continually being discovered in the central nervous system. From their localization and function, some of these peptides appear to be good candidates to have a role in the regulation of biological rhythms. For example, leptin, which is mostly involved in the regulation of food intake (Caro et al., 1996) has binding sites in the mouse pineal gland (Dal Farra et al., 2000); apeline has receptors that are highly expressed in the pineal gland and SCN (De Mota et al., 2000); ghrelin, a peptide involved in the hypothalamic regulation of energy homeostasis (Horvath et al., 2001) may also be a possible candidate.

15. Conclusion: (Neuro)Peptides Are True Pineal Transmitters. Since the 1980s, most studies on pineal peptides have focused either on the immunocytochemical demonstration of their presence and origin in the pineal gland or on their biochemical effects on MEL synthesis (Table 1). All the preceding studies have shown that several peptides of the pineal gland bind to specific receptors to regulate some metabolic pathway(s), especially synthesis of MEL (Table 1). The precise physiological role of these pineal peptides in the regulation of MEL rhythmicity, however, remains to be determined. The observations of daily and seasonal variations in their pineal content associated with specific daily and seasonal modulation of pineal metabolism (for example, the associated variations in NPY content and HIOMT activity: Shinohara and Inouye, 1994; Møller et al., 1998; Ribelayga et al., 1997, 1998c) support a physiological function of these neuropeptides in the expression of the daily and annual MEL rhythms.

To evaluate their function in the pineal physiology, it will be necessary to make timed correlations between the presence/absence/variations of each peptide with a particular situation of pineal metabolism and/or an associated physiological function, and then to prove causality. This will definitely require an expansion of studies to other species, especially those with marked seasonal rhythms. For example, in the European hamster, we have observed that seasonal variations in pineal NPY-IR are associated in time with those of pineal HIOMT activity and MEL and 5-ML concentrations (Vivien-Roels et al., 1992; Møller et al., 1998; Ribelayga et al., 1998c). These in vivo results are very important because for the first time they point to a possible physiological function of a neuropeptide in the mammalian pineal gland.

In addition, in vivo microdialysis experiments with local pineal infusion of neuropeptide agonists/antagonists or antisense molecules for neuropeptide receptors should be continued to investigate the in vivo effect of neuropeptides in physiological conditions. The confirmation, by microdialysis, of a stimulatory effect of locally infused VP on endogenous nocturnal MEL secretion (Barassin et al., 2000) is a good example of our future in vivo studies.

Finally, it will be necessary to determine the nature of the information brought to the pineal gland by the peptides. Do the peptides, like NE, bring photic information about the environment or do they transmit complementary information about other nonphotic environmental factors (temperature, humidity, food quality) or the physiological state of the organism? At present it is not possible to answer these questions. However, it should be borne in mind that the concentrations of numerous peptides of the central nervous system are modulated by nonphotic environmental factors (for example, temperature, food availability). It is thus possible that some of the peptides present in the pineal gland might represent the anatomical and functional way by which nonphotic stimuli reach and are integrated by the pineal gland (Pévet et al., 1986, 1989a; Pévet, 1987).

B. Other Nonadrenergic, Nonpeptidergic Transmitters of the Pineal Gland

In addition to NE and peptides, the metabolic activity of the pineal gland may be regulated by several other neurotransmitters and hormones that have been made the object of earlier reviews (Cardinali, 1979; Ebadi, 1984; Ebadi and Govitrapong, 1986; Cardinali et al., 1987).

1. Serotonin. The pineal gland is characterized by high intracellular levels of 5-HT stored, by vesicular monoamine type 1 transporter, in cytoplasmic vesicles in the long branching processes of pinealocytes (Hayashi et al., 1999).

The 5-HT content in the pinealocytes has generally only been considered as cellular stock used as a substrate for the synthesis of MEL (Mefford et al., 1983; Klein, 1985) because it exhibits a daily rhythm (90 ng/gland during the day and 10 ng/gland at night in the rat; Quay, 1963) opposite to that of MEL. While this function of 5-HT is important, it may not be its only role. Indeed, comparison of the daily MEL and 5-HT rhythms shows, especially at the day/night and night/day transitions, that these two indoles do not vary in a strict opposition in the rat (McNulty et al., 1986), Syrian hamster (Miguez et al., 1995a), Siberian hamster (Miguez et al., 1996), and European hamster (Pévet et al., 1989b). The concentration of 5-HT in the pineal gland decreases markedly at the beginning of the night before AA-NAT activation and MEL release. In addition, in the rat pineal gland the nocturnal decrease in 5-HT (80 ng/gland) is far larger than the nocturnal increase in MEL (1 ng/gland).

Several studies have reported that 5-HT is also a secretory product of the pinealocytes (Shein et al., 1967; Walker and Aloyo, 1985; Chuluyan et al., 1989; Miguez et al., 1997). Furthermore, using pineal microdialysis, it has been shown that 5-HT is released in the pineal extracellular medium during the day with a significant increase at the beginning of the night followed by a marked decrease later in the night (Azekawa et al., 1991; Sun et al., 2002). These observations, suggesting that pineal 5-HT may display auto/paracrine effects on pineal metabolism, have triggered several studies to elucidate the mechanisms regulating 5-HT release and the role of 5-HT in the rat pineal gland (Sugden, 1990a; Olcese and Münker, 1994; Miguez et al., 1997). We have found that there is a high basal release of 5-HT (approximately 10 to 15 ng/h/7 $\times$ 10^4 pineal cells) compared to that of MEL (0.1 to 0.2 ng/h/7 $\times$ 10^4 pineal cells) in cultured pineal cells (Miguez et al., 1997). This is related to high basal TPOH activity since the 5-HT release was strongly inhibited by *p*-CPA, a TPOH inhibitor. Interestingly, *p*-CPA also markedly decreased intracellular 5-HT levels, demonstrating that the latter does not constitute a "passive cellular stock" to be used for MEL synthesis, but a "transitory stock" in constant renewal that is depleted if the synthesis of 5-HT stops. In addition, we have observed that when 5-HT synthesis is inhibited by *p*-CPA, MEL synthesis and release are significantly reduced, even though the intracellular 5-HT levels are still sufficient. These results corroborate in vivo experiments using a similar TPOH inhibition (King et al., 1984).

NE increases 5-HT release via activation of both α_1- and β_1-AR. Activation of α_1-AR-induces a Ca^{2+}-dependent exocytosis of 5-HT per se (Aloyo and Walker, 1987, 1988; Sun et al., 2002; Yamada et al., 2002) and a β_1-AR-induced synthesis and release (Olcese and Münker, 1994; Miguez et al., 1997). The latter result is in agreement with the observation that TPOH activity is increased by administration of a β_1-AR agonist (Ehret et al., 1991). The in vitro release of 5-HT from stimulated rat pinealocytes depends on the metabolic orientation of 5-HT that depends on the level of AA-NAT activity: with moderate β_1-AR stimulation, the synthesis and release of both 5-HT and MEL are increased; following strong β_1-AR stimulation, the intracellular levels and release of 5-HT are markedly decreased while MEL synthesis and release are maximal (Miguez et al., 1997). These in vitro results are in agreement with the observations found using pineal microdialysis (Azekawa et al., 1991; Sun et al., 2002), namely that extracellular 5-HT levels are high during the day, further increased at the beginning of the night, and then markedly decreased during the night because of a major mobilization of 5-HT for MEL synthesis. This triphasic rhythm in 5-HT release is circadian and depends on the NAergic input (Sun et al., 2002).

The putative role of extracellular 5-HT on pineal metabolic activity has been examined. Early studies showed that part of the 5-HT released into the extracellular medium was taken up by the sympathetic nerve endings to be oxidized into 5-HIAL and then metabolized into 5-MIAA and 5-ML in the pinealocytes (Neff et al., 1969; Jaim-Etcheverry and Zieher, 1983; Masson-Pévet and Pévet, 1989). In addition, extracellular 5-HT potentiates MEL secretion induced by β_1-AR stimulation (Sugden, 1990a; Olcese and Münker, 1994; Miguez et al., 1997). We have proposed that this effect may be mediated by activation of 5-HT_2 receptors, although the 5-HT_2 agonist/antagonist concentrations used to obtain a significant effect were quite high (up to 10 μM; Miguez et al., 1997). This 5-HT_2 receptor was first characterized in the bovine pineal gland (Govitrapong et al., 1991) and was recently proposed to be of the $5HT_{2c}$ subtype in the rat pineal gland (Steardo et al., 2000). It would be of interest to confirm these results by studying the second messengers theoretically induced by 5-HT_2 receptor activation, namely Ca^{2+} and IP_3. In support of this it has been reported that 5-HT could induce Ca^{2+} influx in bovine pinealocytes (Cardinali et al., 1991).

In addition to intracellular 5-HT, the rat pineal gland contains 5-HT-containing nerve fibers arising from the

raphe nuclei (Leander et al., 1998). This observation is in agreement with earlier reports showing that after SCGx a number of 5-HT fibers remain in the pineal stalk (Korf and Møller, 1985; Matsuura et al., 1994).

What could the physiological importance of a 5-HT positive autocrine effect be? In the rat pineal gland extracellular 5-HT concentrations increase at the beginning of the night (Azekawa et al., 1991; Sun et al., 2002) when NAergic stimulation is probably still moderated. This transient increase could help to increase the nocturnal stimulation of MEL synthesis. However, it should be noted that while a nocturnal injection of a $5HT_{2C}$ agonist enhances MEL synthesis, a nocturnal injection of a $5HT_{2C}$ antagonist has no effect, indicating a phasic rather than a tonic effect of 5-HT on MEL synthesis (Steardo et al., 2000). In addition, 5-HT could have other effects, especially on pineal vascular flow rate. A presynaptic effect on NE release could also be considered since 5-HT has been reported to display presynaptic effects on neurotransmission in the hippocampus (Matsumoto et al., 1995) and SCN (Pickard et al., 1999).

2. Dopamine. Some observations indicate that DA is not only the precursor of NE, but also a true pineal neurotransmitter. TH-IR and DA β-hydroxylase immunonegative fibers exist in the pineal gland (Jin et al., 1988). In addition, DA concentrations display a marked daily rhythm with higher nocturnal values in the rat, cow, Siberian hamster, and Syrian hamster (Fujiwara et al., 1980; Govitrapong et al., 1989a; Hermes et al., 1994; Miguez et al., 1995a, 1996). Furthermore, after SCGx, TH activity and DA are still detectable in the rat pineal gland (Hernandez et al., 1994). In isolated membranes of the bovine pineal gland, a high density of typical subtype 1 DA receptor (D_1-R) (positively coupled to AC) has been characterized (Simonneaux et al., 1990a). The bovine pineal gland also contains typical subtype 2 DA receptors (D_2-R) (negatively coupled to AC) although with a lower density (Govitrapong et al., 1984). In this species the density of D_1-R is markedly higher (6- to 20-fold) than the density of β_1-AR, α_1-AR, and D_2-R (Simonneaux et al., 1991b), suggesting an important role for DA in the regulation of pineal metabolic activity. Biochemical studies performed in cultured rat pineal glands have shown that DA displays an inhibitory effect at low concentrations (0.1 μM) and a stimulatory effect at high concentrations (10 μM) on AA-NAT activity and MEL release (Axelrod et al., 1969; Govitrapong et al., 1989a), probably related to the presence of the two subtypes of DA receptors. A recent report shows that DA may interfere with α_1-AR to induce Ca^{2+} signaling in the rat pineal gland (Rey et al., 2001). The presence of DA-containing fibers, the identification of specific DA receptors, and the demonstration of biochemical effects of DA suggest that DA may be a pineal neurotransmitter whose physiological role remains to be established.

3. Acetylcholine. Cholinergic fibers have been identified in the pineal gland of several mammals (see Romijn, 1973; David and Kumar, 1978; Phansuwan-Pujito et al., 1990, 1991b, 1999 for reviews). The origin of these pineal cholinergic fibers may be the habenular nucleus or peripheral parasympathetic (pterygopalatine or otic) ganglia. In addition, some cells of the pineal gland (nervous cells and/or pinealocytes) synthesize ACh (Romijn, 1975; Wessler et al., 1997; Phansuwan-Pujito et al., 1999). The ACh content of the pineal gland exhibits a marked daily rhythm with nighttime values being 10- to 20-fold higher than daytime values (Wessler et al., 1997).

The characterization of cholinergic receptors in the pineal gland of some mammals strengthens the idea of parasympathetic modulation of pineal metabolic activity. High-affinity muscarinic receptors (mACh-R) have been characterized in the pineal gland of the rat, sheep, and cow (Taylor et al., 1980; Finocchiaro et al., 1989; Govitrapong et al., 1989b). The presence of nicotinic receptors (nACh-R) has also been demonstrated by immunocytochemistry (in 25% of pineal cells: Reuss et al., 1992b), by autoradiography (Stankov et al., 1993), and by in situ hybridization (indicating the $\alpha_3\beta_2$ composition of the nACh-R; Wada et al., 1989; Yeh et al., 2001).

Various postsynaptic effects of activation of mACh-R have been postulated: stimulation of 5-HT synthesis and release without any effect on MEL (Finocchiaro et al., 1989); stimulation of PI hydrolysis and MEL production (2-fold) via cAMP-independent mechanisms (Laitinen et al., 1989, 1992); inhibition of AA-NAT activity (Phansuwan-Pujito et al., 1991a); increase in the number of pineal synaptic ribbons (Gupta et al., 1991), and an increase in $Ca^{2+}{}_i$ (Marin et al., 1996). The main effect of mACh-R activation, however, probably occurs at the presynaptic level. A presynaptic effect was first postulated following the observation of an effect of ACh on the whole pineal gland but not on cultured pinealocytes (Laitinen et al., 1995). This hypothesis has now been confirmed by pineal microdialysis showing that carbachol inhibits the production of NAS and MEL via presynaptic inhibition of NE release (Drijfhout et al., 1996a).

Activation of the postsynaptic nACh-R induces, in a large majority of rat pinealocytes, Ca^{2+} influx via L-type Ca^{2+} channels following membrane depolarization (Schomerus et al., 1995; Letz et al., 1997). In addition, it has been shown that nicotine has no effect by itself but inhibits NE-induced MEL secretion (Stankov et al., 1993). It has been proposed that nACh-R-induced cell depolarization leads to the release of Glu from pineal microvesicles (MV), which in turn inhibits the secretion of MEL (Kus et al., 1994; Letz et al., 1997; Yamada et al., 1998b; see below). Interestingly, recent studies reported a developmental switch from rat pineal mAChR to nAChR around the third week of life with the parallel appearance of L-type Ca^{2+} channels (Schomerus et al., 1999; Wagner et al., 2000). In adult bovine pineal cells activation of either nACH-R or mACh-R induces an in-

crease in intracellular level of Ca^{2+}, but with no apparent effect of basal or NE-induced AA-NAT activity and MEL synthesis (Schomerus et al., 2002).

In summary, parasympathetic input would therefore exert a tonic inhibition on pineal activity, on the one hand via presynaptic inhibition of NE release and, on the other hand, via postsynaptic activation of the inhibitory intrapineal Gluergic system.

4. Glutamate. Glu, usually considered to be an excitatory amino acid, is present in the pineal gland at high concentrations (1.2 mg/g rat pineal). It is mainly localized in pinealocytes, associated with MV (the endocrine counterpart of synaptic vesicles), but it has also been found in glial cells and fibers whose origin is unknown (McNulty et al., 1992; Redecker and Veh, 1994). It has been suggested that the pineal Glu concentration is partly controlled by NE (McNulty et al., 1992). The transport of Glu in MV and its effect on pineal metabolic activity has been well studied in several mammals (Govitrapong and Ebadi, 1988; McNulty et al., 1992; Kus et al., 1993, 1994; Redecker and Veh, 1994; van Wyk and Daya, 1994; Moriyama and Yamamoto, 1995a,b; Yamada et al., 1996a,b, 1997b, 1998a,b). Glu is taken up into both pinealocytes (Yamada et al., 1997b) and interstitial cells (Redecker and Pabst, 2000) mainly via a type 1 Na^+-dependent Glu transporter and then stored in MV via the synaptic vesicle protein of type 2 (SV2B, Hayashi et al., 1998). Following cell depolarization, Glu is released by exocytosis via Ca^{2+}-dependent mechanisms. The endogenous transmitter responsible for depolarization-induced Glu release could be ACh acting via nACh-R (Letz et al., 1997; Yamada et al., 1998a). Extracellular Glu inhibits AA-NAT activity and MEL secretion induced by NAergic stimulation. In the rat pineal gland the binding site for Glu is a class II metabotropic Glu receptor of type 3 (mGluR3) coupled to a G_i protein responsible for the cAMP-dependent decrease in AA-NAT activity and MEL synthesis (Yamada et al., 1998b). The class I mGluR5 receptor is also present in pineal cells and triggers Ca^{2+} efflux from intracellular stores (Yatsushiro et al., 1999; Pabst and Redecker, 1999). Other ionotropic Glu receptors have also been reported in the pineal glands of several species (Sato et al., 1993; Govitrapong et al., 1986; Mick, 1995; Yatsushiro et al., 2000). In the rat pineal gland GluR1 is functionally expressed in pinealocytes and may participate in a Ca^{2+}-signaling cascade that enhances and expands the Gluergic signal throughout the pineal gland (Yatsushiro et al., 2000). Glu has also been proposed to inhibit HIOMT activity, but not HIOMT mRNA (Ishio et al., 1999). It is interesting to note that Glu also activates NOS in several tissues and could therefore be involved in cGMP synthesis. In addition, it has been shown that Glu can regulate the presynaptic release of NE (Wang et al., 1992).

Apart from Glu, L-aspartate is present in high concentrations in the rat pineal gland; it is released together with Glu during exocytosis and inhibits the NE-induced increase in AA-NAT activity and MEL synthesis (Yamada et al., 1997a; Yatsushiro et al., 1997). Of any mammalian tissue, the highest concentrations of D-aspartate occur in the pineal gland (Imai et al., 1995; Lee et al., 1997; Schell et al., 1997). D-aspartate is actively taken up by the pineal cells and then released upon NE-stimulation, where it strongly inhibits the NE-induced increase in AA-NAT activity and MEL synthesis (Ishio et al., 1998; Takigawa et al., 1998). In addition to Glu and aspartate, cultured pinealocytes also release glycine upon stimulation with depolarizing concentrations of KCl (Redecker et al., 2001).

These data show that the amino acid Glu (and possibly aspartate) is probably an important auto/paracrine transmitter involved in the regulation of MEL synthesis in the pineal gland. In vitro, it appears to be released upon ACh stimulation and inhibits NE-induced MEL synthesis. In addition, the glutamatergic communication in the pineal gland may enable paracrine cross-talk among pinealocytes as well as interactions between pinealocytes and interstitial cells. Additional in vivo experiments are now needed to clarify the exact role of this amino acid negative loop in the regulation of MEL synthesis.

5. GABA. GABA, an inhibitory neurotransmitter, is present in the pineal gland of several mammals where it is considered to be an intrapineal transmitter with paracrine effects (Ebadi and Chan, 1980; Ebadi and Govitrapong, 1986; Rosenstein et al., 1989a,b, 1990, 1991). The immunodetection of GABA transporters (GAT 1–3) in pinealocytes, and to a lesser extent in interstitial cells, together with the GABA synthesizing enzyme confirms the paracrine function of GABA in the gerbil pineal gland (Redecker, 1999). GABA has also been observed in the pinealopetal fibers that remain after SCGx and seen passing through the posterior and habenular commissures and the deep pineal gland, both observations indicating a central origin of this innervation (Sakai et al., 2001).

Typical A-type ($GABA_A$-R) and B-type ($GABA_B$-R) GABA receptors have been identified in the pineal gland. In the rat pineal gland, GABA inhibits NE-induced MEL synthesis via $GABA_A$-R and inhibits the NE release via $GABA_B$-R (Rosenstein et al., 1989a, 1990). In the bovine pineal gland GABA decreases NAergic stimulation of AA-NAT activity, increases Cl^- flux, and decreases 5-HT release (Ebadi and Chan, 1980; Rosenstein et al., 1989b). In the sheep pineal gland, GABA also inhibits the NE-induced increase of AA-NAT activity (Foldes et al., 1984). The quantity of GABA in the pineal gland of the rat (Waniewski and Suria, 1977) and Syrian hamster (Kanterewicz et al., 1993) exhibits a daily variation with higher nighttime values.

6. Taurine. Taurine is an amino acid that displays high concentrations in the pineal gland (LaBella et al., 1968; McNulty et al., 1992). Its release from the pineal

gland is stimulated by NE (Wheler and Klein, 1980). Taurine stimulates AA-NAT activity and MEL synthesis, but this effect may not be specific because it can be inhibited by the β_1-AR antagonist, PROP (Wheler et al., 1979).

7. Histamine. Pineal gland of various mammalian species contains histamine (Quay, 1974). The rat pineal gland is moderately innervated by histaminergic fibers of central origin (Mikkelsen et al., 1992). Histaminergic neurons of the tuberomammillary nucleus of the posterior hypothalamus project via the posterior commissure to the deep pineal gland, the pineal stalk, then to the proximal part of the superficial pineal gland. In the chicken, histamine is a powerful stimulator of cAMP (Nowak et al., 1997), but in the rat no effect has been observed on AA-NAT activity (Buda and Klein, 1978) or the metabolism of PI (Muraki, 1972). The possibility that a metabolite of histamine may have an effect needs to be considered, since enzymes involved in the metabolism of this amine are present at high concentration in the pineal gland (Quay, 1974). In addition, a presynaptic effect of histamine on the release of a neurotransmitter, especially NE, is possible (Hill, 1990; Yamazaki et al., 2001).

8. Adenosine and ATP. In the autonomic nervous system, ATP is coreleased with NE (Burnstock, 1976). In the rat pineal gland, NE release is accompanied by a release of ATP that is subsequently metabolized into adenosine by the pineal cells (Nikodijevic and Klein, 1989). ATP and its metabolite adenosine exert their effect via two main families of purine receptors: P_1-type receptor (P_1-R; former nomenclature grouping the A_1, A_{2a}, A_{2b}, and A_3 adenosine receptor subtypes) coupled to G-proteins and P_2-type receptors (P_2-R; specific receptor to ATP) including P_{2X}-R (ligand-gated ion channels) and P_{2Y}-R (G-protein-coupled). In the rat pineal gland activation of adenosine receptors elevates cAMP levels (Sarda et al., 1989) and increases AA-NAT activity and MEL synthesis (Gharib et al., 1989; Nikodijevic and Klein, 1989; Vacas et al., 1989; Ferreira et al., 1994). In addition, ATP binds to P_{2Y}-R to activate PLC and therefore potentiates the NE-induced synthesis of MEL (Gharib et al., 1992; Stehle et al., 1992; Nicholls et al., 1997; Mortani Barbosa et al., 2000; Ferreira and Markus, 2001). The sheep pineal gland is reported to possess adenosine receptors, activation of which induces different effects on MEL synthesis according to the dose of agonist (Falcon et al., 1997).

9. Nitric Oxide. NO is a diffusible neurotransmitter implicated in a variety of neuroendocrine processes. Three isoforms of the synthesizing enzyme NOS have been described: type I is neuronal, Ca^{2+}-dependent, and not inducible; type II is Ca^{2+}-independent and inducible; type III is the endothelial isoform (Jacobs et al., 1999).

NO is synthesized in the sympathetic fibers innervating the pineal gland of sheep (high density of neuronal NOS; Lopez-Figueroa et al., 1996) and rat (presence of NADPH-diaphorase activity; Lopez-Figueroa and Møller, 1996). NO is also synthesized in nonsympathetic (VIPergic) fibers of the sheep and rat pineal gland (Lopez-Figueroa and Møller, 1996; Lopez-Figueroa et al., 1997). In addition, neuronal NOS is present in the rat (Lin et al., 1994; Schaad et al., 1994, 1995a; Lopez-Figueroa and Møller, 1996) and cow (Maronde et al., 1995) pinealocytes (but not in the sheep: Lopez-Figueroa et al., 1996). Although NO appears to be synthesized only in a small subpopulation of pineal cells, it is thought to be an intercellular messenger acting on all pineal cells (Spessert et al., 1998). Pineal NOS expression and activity are regulated by NE in the long-term/photoperiodic range (see *Section V.A.7.*; Schaad et al., 1994; Jacobs et al., 1999; Spessert and Rapp, 2001). In addition, NOS activity, measured by NADPH-diaphorase activity, is present in the endothelial cells of the pineal blood vessels (Lopez-Figueroa and Møller, 1996; Lopez-Figueroa et al., 1996).

Different roles of the diffusible factor on pineal activity have been suggested. NO is involved in NE-induced cGMP synthesis (see *Section V.A.2.*; Spessert et al., 1993; White and Klein, 1993; Lin et al., 1994). In addition, NO could be involved in the release of neurotransmitters such as VIP and NE, as already shown in some tissues (Lonart et al., 1992). However, we did not observe any significant effect of NO donors or NOS inhibitors on the presynaptic release of NE (Simonneaux and Schaad, unpublished results). NO could also be involved in the regulation of pineal blood flow (most VIPergic fibers end in pineal perivascular spaces; NO is known to display vasorelaxant effects, similar to VIP; and NOS activity has been measured in the endothelium of blood vessels in the sheep and rat pineal gland). Finally, exogenous NO is reported to be a powerful inhibitor of MEL synthesis in the rat and bovine pinealocytes (Maronde et al., 1995) via cGMP-independent mechanisms that remain to be determined. Similarly, the spontaneous electrical activity of rat pinealocytes is inhibited by exogenous NO (Schenda and Vollrath, 1997).

10. Gonadal Steroids. Endogenous MEL is involved in the regulation of reproductive function of photoperiodic species (Reiter, 1980, 1993). Interestingly, gonadal steroids may exhibit a feedback effect on the pineal gland (see Cardinali, 1979 for review).

The pineal gland specifically accumulates estradiol and testosterone (Nagle et al., 1972, 1974) and contains nuclear binding sites for estradiol, testosterone (Cardinali, 1977; Cardinali et al., 1983; Moeller et al., 1984), 5α-dihydrotestosterone (Cardinali et al., 1974a; Gupta et al., 1993) and progesterone (Vacas et al., 1979). The number of cytoplasmic estrogen receptors and translocation of the hormone/receptor complex to the nucleus are partly regulated by NE (Cardinali et al., 1975, 1983) and 5α-dihydrotestosterone receptor expression is increased by NE (Gupta et al., 1993).

Large changes in sex steroid levels alter pineal metabolism, and these effects are different in males and females (Hamill et al., 1984; Hernandez et al., 1990; Alonso et al., 1995; Yie and Brown, 1995). In general, testosterone exhibits stimulatory effects and castration reduces cAMP concentrations (Karasek et al., 1978), AA-NAT activity (Rudeen and Reiter, 1980), and MEL synthesis (Hernandez et al., 1990). In contrast, estradiol displays an inhibitory effect on the α_1/β_1-AR-induced increase in cAMP and $Ca^{2+}{}_i$ levels, AA-NAT activity, and MEL production in female rats, while ovariectomy leads to a significant increase in the cAMP/AA-NAT/MEL pathway (Moujir et al., 1990a; Okatani et al., 1997, 1998; Hayashi and Okatani, 1999; Ishizuka et al., 2000; Hernandez-Diaz et al., 2001). Similarly, an increase in nocturnal MEL secretion (associated with an increase in AA-NAT but not HIOMT activity) was observed during menopause in relation to the existence of a low estrogen environment in the rat (Okatani et al., 1999) and human (Okatani et al., 2000). It should be noted, however, that in the female guinea pig, in contrast to the rat, physiological doses of estradiol (10 to 100 nM) increased cAMP accumulation and MEL release (Cardinali et al., 1986). Progesterone injection for 2 weeks did not produce any significant change in MEL (Okatani et al., 1997). The putative effect of the sex steroids on HIOMT activity is controversial. In female rats, a number of studies reported no effect on HIOMT activity (Yuwiler, 1985, 1989; Okatani et al., 1998, 1999), whereas others found various effects (Wurtman et al., 1965; Alexander et al., 1970; Houssay and Barcelo, 1972; Nagle et al., 1972; Preslock, 1977). One of the first in vitro studies showed that HIOMT activity in castrated female rats was stimulated (in 2 h) by physiological doses of estradiol, an effect that was abolished in the presence of RNA and protein synthesis inhibitors (Mizobe and Kurokawa, 1976). In male rats, castration decreased and testosterone increased HIOMT activity (Nagle et al., 1974). Progesterone inhibits HIOMT activity and MEL secretion (Cardinali et al., 1976, 1986) but this finding remains controversial (Alonso et al., 1993).

Until now it has been difficult to evaluate the effect(s) of endogenous gonadal steroids on pineal metabolism because of the high interanimal variations. In vivo variations in the activity of the pineal gland depending on the female sexual cycle have been observed in several species: rat (Quay, 1963; Wurtman et al., 1965; Ozaki et al., 1978), sheep (Cardinali et al., 1974b), mole (Pévet and Smith, 1975), human (Wetteberg et al., 1976; Parry et al., 1990), squirrel (Ellis and Balph, 1976), and pony (Wesson et al., 1979), with no consistent pattern according to the estrous stage. In addition, the effect of large changes in steroid levels following gonadal suppression or steroid injections are not considered to be physiologically relevant. Therefore, we recently performed a detailed analysis of AA-NAT and HIOMT gene expression and enzyme activity and MEL content and release in the pineal gland of female rats throughout the estrous cycle. We found no estrous stage-dependent differences in pineal AA-NAT and HIOMT gene expression and activity or in the MEL content. This was confirmed by a 5- to 6-consecutive-day pineal microdialysis of cycling female rats where none of the animals showed a significant variation in endogenous melatonin release with different estrous stages (Skorupa et al., 2003).

The above data suggest that the MEL rhythm is not altered by the estrous cycle in normal female rats. However, marked changes in the circulating steroid levels (steroid injection, castration, menopause) have been reported to alter MEL synthesis and release.

VII. General Conclusions and Perspectives

The present review outlines the extraordinary capacity of the pineal gland to integrate numerous hormonal and neural messages via several signal transduction pathways. It has been proven, at least in the rat, that the SCN clock-driven nocturnal NAergic stimulation is essential for the generation of the circadian rhythm of pineal MEL synthesis and release. Other (neuro)transmitters are present in the pineal gland to refine this NAergic input. We have shown that this is an important function for some of the neuropeptides present in the pineal gland. However, to date most of these studies have been performed in vitro and/or in an acute experiment. Evaluation of the role of these non-NAergic pineal transmitters in in vivo conditions is thus definitely needed. The development of new technologies will soon allow the necessary in vivo investigations. Future studies should focus on characterization of 1) the endogenous release and effects of the various pineal transmitters (for example, using pineal microdialysis, in vivo infusion of specific antisense oligonucleotides, or genetically modified animals); and 2) the mechanisms involved in the photoperiodic/seasonal plasticity displayed by the pineal gland (seasonal plasticity of the neural pathways afferent to the pineal gland, analysis of seasonal variation in gene expression using the microarray technology, and use of genetically modified animals). The use of these tools will first require a better knowledge of the genome of photoperiodic rodents, for example, hamsters. This development is now technically possible and promises to open an exciting new approach to our understanding of how seasonal information is integrated to shape the MEL message and subsequently control the physiology of the entire organism.

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