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The Rhythm and Blues of Gene Expression in the Rodent Pineal Gland

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In all vertebrates, melatonin is rhythmically synthesized in the pineal gland and functions as a hormonal message, encoding for the duration of night. In rodents, the nocturnal rise and fall of the arylalkylamine *N*-acetyltransferase (AA-NAT) activity controls the rhythmic synthesis of melatonin. This rhythm is centered around the transcriptional regulation of the AA-NAT by two norepinephrine-inducible transcription factors, the activator CREB (Ca^{2+}/cAMP-response element binding protein) and the inhibitor ICER (inducible cAMP early repressor). CREB is activated by phosphorylation, which is one of the fastest responses in pinealocytes upon adrenergic stimulation, occurring within minutes. ICER in turn accumulates only after several hours, a time gap resulting from the required *de novo* protein synthesis upon adrenergic stimulation. However, these molecular components of neuroendocrine signaling in the rodent pineal gland are supplemented by the impact of a variety of neurotransmitters and neuromodulators, and by translational and post-translational mechanisms. By molecular crosstalk, those different inputs on pinealocytes seem to fine-tune the shape of the melatonin signal, by interacting at various levels with the NE/cAMP/pCREB/ICER pathway. In addition, these alternate signaling routes may be important in acute "emergency" situations. Together, concerted signaling events in the rodent pineal gland help to generate a stable and reliable hormonal message of darkness for the body, that, however, can be altered rapidly upon sudden and unexpected "error" signals.

Key Words: Melatonin; rhythm; clock genes; CREB; ICER; AA-NAT.

Introduction— or, the Need for Rhythm Coordination

An orchestra can play the harmonies of the blues only as well as instruments are tuned and swinging in phase—or in antiphase. Most important, a good sound depends on how well the rhythm is guided by the conductor. When listening to individual players in the orchestra of the mammalian pineal gland (by morphology, electrophysiology, biochemistry, immunohistochemistry, *in situ* hybridization, and microarray), the music is remarkably tuned. This observation tells us that a common conducting driving force provides a stringent measure, following the score of the daily light and dark regimen, and/or that the phylogenetic pressure has formed a homogeneous swinging group of instrumentalists to provide the "audience" of body cells with well-framed cues from environmental changes in the ambient lighting conditions. This review will focus on our current knowledge of gene regulation in the rodent pineal gland, and speculate on the role of rhythmically expressed genes with as yet unknown function.

For all living organisms, a fundamental requirement to survive is the adaptation to and the anticipation of changing external light conditions. This is particularly important for mammals, to cope properly with season and, consequently, to ensure a timely mating and delivery of offspring. These basic demands are managed by the so-called photoneuroendocrine system (PNS), consisting of the retina, the suprachiasmatic nucleus of the hypothalamus (SCN), and the pineal gland *(1)*. Central to the PNS is the SCN, which generates a circadian (*circa*: about; *dies*: day) rhythm through the molecular interplay of clock gene products, regulating their own transcription in a feedback loop *(2)*. The free-running endogenous oscillator in the SCN is entrained on a daily basis to changing external lighting conditions through retinal information, transmitted via the retinohypothalamic tract (RHT) *(3)*. The SCN subsequently conveys the signals from the retina to the pineal gland via a multisynaptic neuronal chain *(4,5)*, with the final stretch formed by postganglionic nerve fibers, originating from the superior cervical ganglia (SCG). The phasic release of norepinephrine (NE) from these fibers *(6,7)* ensures proper translation of clock time into the rhythmic melatonin synthesis. The hormone is not stored within pinealocytes, but is directly

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released into the circulation upon its formation. Melatonin thus mirrors low activity of the SCN during night-time.

Notably, the nocturnal elevation of melatonin is a general feature in the pineal gland of all vertebrates *(1)*, and it is thus not surprising that the steps involved in its synthesis are similar. Hormone production starts with the uptake of the amino acid tryptophan from the blood into pinealocytes and subsequent hydroxylation by the tryptophan hydroxylase to 5-hydroxytryptophan. In the next step, serotonin (5-hydroxytryptamin) is produced via decarboxylation of 5-hydroxytryptophan, serving as a substrate for the arylalkylamine *N*-acetyltransferase (AA-NAT), which catalyzes the formation of *N*-acetylserotonin. In the ultimate step, hydroxyindole-*O*-methyltransferase (HIOMT) converts *N*-acetylserotonin into melatonin. AA-NAT is a key enzyme in melatonin synthesis, and its activity levels may be controlled at different stages of its synthesis and processing, namely (1) at the transcriptional level, (2) through posttranscriptional processes, such as phosphorylation and binding to chaperone proteins, and (3) by regulation of protein degradation velocity by proteosomal proteolysis *(8)*.

In the rodent pineal gland, the nocturnal increase of melatonin synthesis is pivotally regulated on the *Aa-nat* expression level and is dependent on the NE-induced activation of the adenylate cyclase/protein kinase A (AC/PKA) pathway, which results in the phosphorylation of CREB (calcium/cAMP response element binding protein) *(9–13)*. The activation of this pathway at the beginning of the dark period is indispensable for the stimulation of melatonin synthesis in rodents.

The Melatonin Message of Darkness— A Constant Sound Through Evolution

Despite its small size, the mammalian pineal gland has the remarkable capacity to transduce various neuronal and hormonal input signals into an output message, common to all vertebrates: regardless of whether a given species is active during daytime (diurnal) or night-time (nycthermal), melatonin synthesis and release into circulation is high during the dark period. Such a hormonal message of darkness has been proven to be advantageous for many species, whose reproduction is related to the solar cycle. Although a rhythmic melatonin synthesis is conserved across vertebrate phyla, molecular mechanisms for generating this cycling pineal output are notably different *(14)*.

In most non-mammalian vertebrates the melatonin-producing pineal organ is directly light sensitive and capable of generating a circadian rhythm *(1)*. These two functions appear to be lost in the course of evolution. The mammalian pineal gland is now driven by neuronally transmitted rhythmic signals from the clock in the SCN. The SCN clockwork is phase-adjusted to changes in the environmental lighting conditions by retinal input. Persisting as an effector of the photoneuroendocrine system, the mammalian pineal has adapted to transduce information from the central clock into the rhythmic production and secretion of the lipophilic melatonin during night time.

The melatonin rhythm is essential for mediating time cues that (1) control seasonal reproduction *(15)*, (2) entrain the SCN to ensure synchrony with the environment in maternal–fetal communication *(16)*, (3) time human physiology and are affected in certain pathologies (sleep–wake cycle, shift-work, rapid travel across several time zones, seasonal affective disorders) *(17, 18)*, and (4) regulate photopigment disc shedding, phagocytosis, and dopamine release from amacrine cells *(19)*. All these effects of melatonin are elicited by G protein–coupled MT1 or MT2 melatonin receptors that are highly concentrated in the SCN and the hypophyseal pars tuberalis, and to a lesser extent in the retina and brain, respectively *(16,20–22)*.

Pineal Neurotransmitters— Conducted Individual Musicians

The phylogentic loss of the direct light sensitivity in mammalian pinealocytes is compensated by a multitude of neurotransmitter/neuromodulators that can inflict on biochemical pathways involved in melatonin synthesis (Fig. 1). Despite—or because of—this multitude a clear hierarchy in impact potency has evolved.

Norepinephrine—The First Violin

The sympathetic neurotransmitter NE is central for rhythmic pineal melatonin synthesis in mammals. In rodents, the nocturnally elevated release of NE from postganglionic nerve fibers *(6)* that originate from the SCG affects pineal physiology through activation of two subtypes of adrenergic receptors (ADR), namely the α_1-ADR and the β_1-ADR. The β_1-ADR is linked via a stimulatory G protein (G_s) to the AC (Fig. 1). Using cultured rat pineal glands or pinealocytes, β_1-ADR activation was shown to cause an approx 6–10-fold increase of intracellular cAMP levels *(23)*. Neither in ex vivo *(9)* nor in in vitro experiments *(23)* does the sole activation of the α_1-ADR affect cAMP levels or melatonin synthesis, but leads to an activation of the phospholipase C and a subsequent release of Ca^{2+} from intracellular IP_3-sensitive stores *(24)*. This increase in Ca^{2+} concentration is followed by an additional influx of Ca^{2+} through membrane-bound calcium-dependent ion channels *(24–26)*. Upon concomitant stimulation of α_1- and β_1-ADRs, the dramatic rise in intracellular Ca^{2+} levels potentiates the activation of the AC by a PKC/calcium/calmodulin dependent kinase (CaMK) *(27,28)* and causes an approx 100-fold increase of intracellular cAMP levels *(23,29,30)*.

The α_2-ADR has also been demonstrated in the rat pineal gland *(30)*. Several in vitro and in vivo studies suggested a presynaptic localization of this receptor on sympathetic nerve terminals, where it may potentially inhibit NE release *(31–34)*. Further, in vitro studies using specific ago-

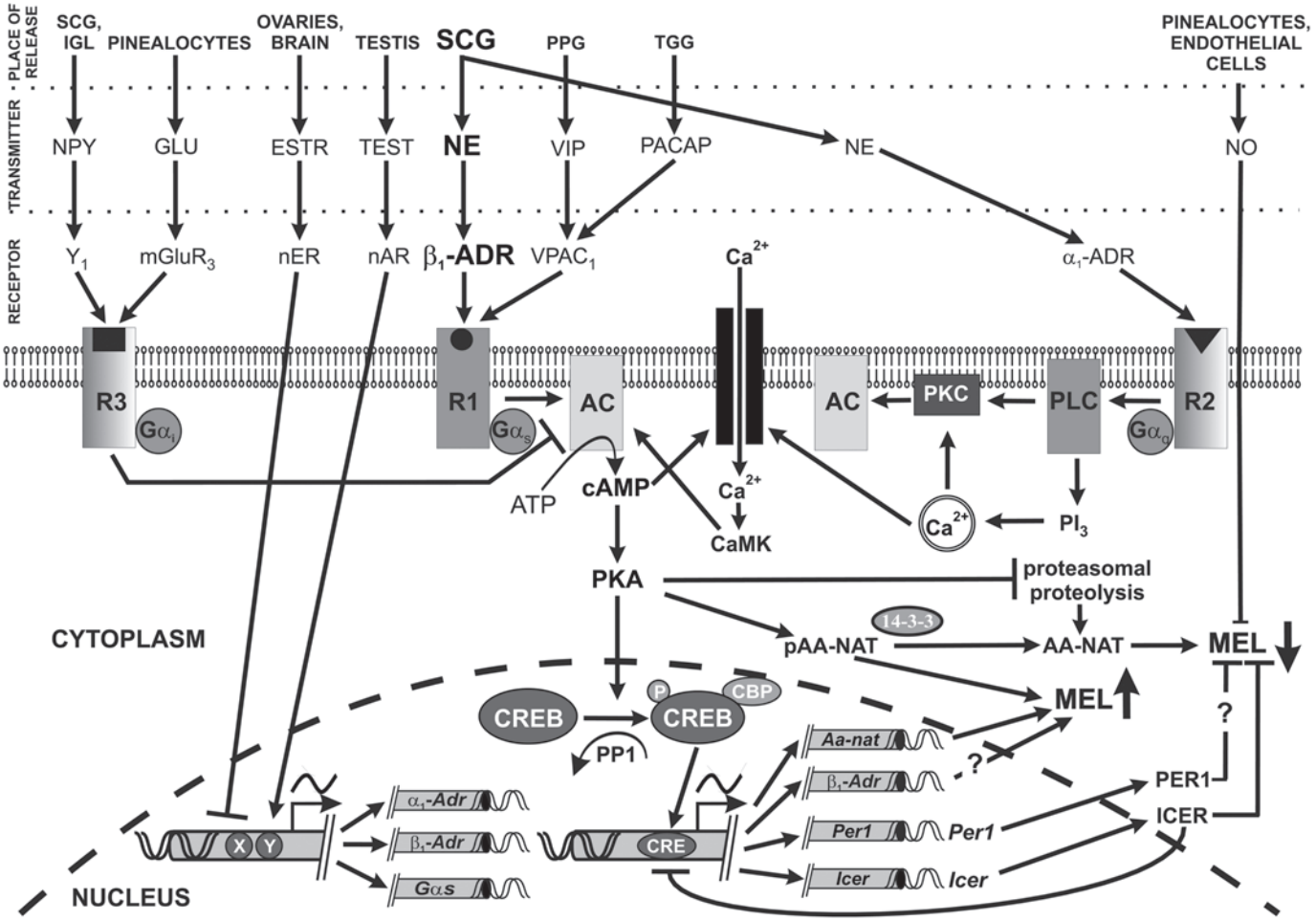

Fig. 1. Important intracellular pathways in the rodent pineal gland. For details see text. Abbreviations: AA-NAT, arylalkylamine *N*-acetyltransferase; AC, adenylate cyclase; ADR, adrenergic receptor; CBP, CREB binding protein; CRE, calcium/cAMP response element; CREB, CRE binding protein; E, estradiol; G, G protein; GLU, glutamate; ICER, inducible cAMP early repressor; IGL, thalamic intergeniculate leaflet; MEL, melatonin; nAR, nuclear androgen receptor; nER, nuclear estrogen receptor; NE, norepinephrine; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase activating peptide; Per1, Period1; PKA, protein kinase A; PKC, protein kinase A; PLC, phospholipase C; PP1, protein phosphatase type I; PPG, pterygopalatine ganglion; SCG, superior cervical ganglion; T, testosterone; TGG, trigeminal ganglia; VIP, vasoactive intestinal polypeptide; R1-3, G protein–coupled receptors.

nists showed the presence of the α_2-ADR in the membrane of pinealocytes and its involvement in the potentiation of AA-NAT activity *(30)* and melatonin release *(35)*.

PACAP and VIP

The stimulatory activity of the neuropeptides PACAP and VIP on rodent pineal melatonin synthesis is well investigated *(36,37)*. The PACAPergic innervation originates from the trigeminal ganglia *(38)*, while VIPergic pinealopetal nerve fibers seem to arise from perikarya in the pterygopalatine ganglia *(39)*. Both transmitters bind to the G_S-coupled $VPAC_1$ receptor *(40)* (Fig. 1), leading to the activation of the AC/PKA/pCREB pathway and a subsequent increase of AA-NAT activity *(9,36,41,42)*. Notably, $VPAC_1$-induced responses on melatonin synthesis and on CREB phosphorylation are several times lower in magnitude than those induced by NE *(36,43)*. This difference was shown to be due to restricted number (50–60%) of VIP/PACAP–responsive pinealocytes *(36)*. Interestingly, VIP and PACAP seem to potentiate NE-dependent melatonin synthesis *(44)*, a situation that is similar to the α_1-ADR–induced potentiation of the β_1-ADR effect. The presumed importance of a peptidergic modulation of melatonin synthesis is confirmed by the fact that in the rat pineal gland the VIP content is threefold elevated at night time as compared to daytime values *(45)*.

NPY

In the rat pineal gland neuropeptide Y (NPY) is co-stored with NE in sympathetic nerve terminals and consequently is co-released with the sympathetic neurotransmitter at night time *(46)*. Some NPYergic nerve fibers seem to originate also from neurons of the thalamic intergeniculate leaflet (IGL) *(47)*. Two subtypes of NPY receptors, Y_1-R and Y_2-R, have been identified to affect synthesis of melatonin.

Both of them are coupled to a G_i protein and their activation results in a reduced activity of the AC *(48)*. Y_2-R is localized presynaptically and inhibits the release of NE induced by high K^+ depolarization by about 45% *(32)*. Postsynaptically, NPY decreases via the Y_1-R the adrenergically induced cAMP levels *(32,49,50)* and the NE-stimulated melatonin synthesis and release *(49,51)*. These in vitro results in rat are supported by in vivo observations, reporting a decrease of AA-NAT activity after NPY injection during the night *(52)*. Other groups *(32,53)*, however, have shown that the postsynaptic NPY action stimulates basal melatonin release and potentiates NE-stimulated melatonin synthesis. This effect was suggested to be due to an increase in intracellular Ca^{2+} concentrations and an effect on HIOMT activity *(54,55)*. Investigations with hamsters suggest that NPY, whose levels are elevated during winter time *(56)*, might also play a role in the annual regulation of melatonin synthesis *(57)*. Again, this NPYergic effect seems to target the HIOMT enzyme.

L-Glutamate

The transmembrane translocation of glutamate (GLU) from the interstitial compartment and/or from the vasculature into pinealocytes and pineal glia cells is achieved by glutamate transporter GLT-1 *(58)*. GLU is subsequently stored in microvesicles by the synaptic vesicle protein of type 2 *(58)*. So far there is no evidence for a day/night difference in uptake, storage, or release of GLU from rodent pinealocytes *(59)*. A secretion of GLU from pinealocytes occurs under Ca^{2+}-regulated exocytosis *(60–62)* and leads to a decrease in melatonin synthesis, due to the presence of metabotropic GLU receptor 3 (mGluR3) in the membrane of pinealocytes. Its activation causes a G_i-mediated and cAMP-dependent decrease in AA-NAT activity *(63)*. Additionally, the ionotropic GluR1 has been shown to trigger Ca^{2+}-evoked exocytosis of GLU from microvesicles in the rat pineal gland, possibly enhancing and expanding the GLU effects on melatonin biosynthesis *(64)*. Another metabotropic receptor, mGluR5, is also expressed in rat pinealocytes *(65)*. Its physiological role is not yet fully understood; however, activation of this receptor is not followed by decrease of intracellular cAMP levels and melatonin synthesis *(65)*.

Interestingly, stimulation of rat pinealocytes with acetycholine (ACh), potently inhibiting melatonin synthesis, also causes the release of glutamate *(63)* by a Ca^{2+}-dependent membrane depolarization *(61)*. As nicotinic ACh receptors are present in the majority of pinealocytes *(66–68)*, their activation seems to trigger GLU exocytosis, which inhibits in a paracrine fashion pineal AA-NAT activity. In this context it is interesting that the intrapineal ACh content in vivo shows a marked daily rhythm, with higher levels of this neurotransmitter at night time (10–20-fold), than during day time *(69)*, underlining the possible cholinergic modulatory role on melatonin biosynthesis.

These data show that GLU might participate in an auto- and paracrine regulation of melatonin synthesis and release; however, additional studies on the mechanisms leading to the release of GLU and its role in the down-regulation of melatonin synthesis in vivo are necessary.

Gonadal Hormones

In photoperiodic species, like hamster and sheep, melatonin is the cue to regulate the activity of the hypothalamic/hypophyseal/gonadal axis. Therefore, it is not surprising that gonadal steroids can feed back on pineal function. The gonadal hormones testosterone (TEST) *(70,71)* and estradiol (ESTR) *(72)* have been shown to affect pineal gland metabolism.

TEST stimulates in vitro intracellular cAMP concentrations *(73)*, AA-NAT activity *(74)*, and also in vivo melatonin synthesis *(71)*.

In contrast, ESTR inhibits the adrenergically induced increase of cAMP and Ca^{2+} levels, of AA-NAT activity and melatonin synthesis *(75)*. All these effects are reversed by ovariectomy *(76–79)*. The classical mechanism of ESTR action is mediated via nuclear receptors that function as TFs. Indeed, in the female rat pineal gland such a direct effect on pineal transcription machinery was suggested, as the ESTR effect on NE-induced cAMP accumulation was not mimicked by a membrane-impermeable form of this steroid *(75)*.

In rat, no estrous stage–dependent differences in pineal AA-NAT and HIOMT activity, or in melatonin content and release were observed *(80)*. It has to be considered, however, that marked changes in circulating steroid levels, induced by castration, by ovariectomy, or by menopause have been reported to alter melatonin levels *(81)*. These data suggest that estrogen does not play a role in the regulation of melatonin levels in fertile female rat, but the steroid might be involved in the changes of melatonin content correlated with aging.

Nitric Oxide (NO)

The detection of neuronal NO synthetase (nNOS) in rodent pinealocytes suggested a function for this diffusible neurotransmitter as an intercellular messenger *(82)*. Indeed, external donors of NO decrease melatonin synthesis in vitro *(83)*. As a source for NO, pinealocytes, but also sympathetic *(84)* and non-sympathetic, VIPergic *(82)* fibers were delineated by NOS immunoreactivity. In addition, NOS was found in endothelial cells of pineal blood vessels *(82,84)*, suggesting NO effects via its known function as a vasorelaxant *(85)*. The mechanism of NO to inhibit melatonin synthesis is still not clear, because adrenergic activation of NOS *(86)* does not decrease melatonin synthesis in vitro, whereas external donors inhibit hormone production *(83)*. Additionally, inhibition of intrinsic NOS does not elevate melatonin synthesis *(86)*. This paradox may be solved by the suggestion that NO synthesized within pinealocytes can indeed

Table 1
Transcriptional Factors, Known to Take Part in the Regulation of Pineal Gene Expression

Transcription factor	Specific features	Binding site	Effect	References
CREB	Activator; nocturnal phosphorylation	CRE	Stimulation of *Aa-nat, β_1-Adr, ICER, Per1* expression at the beginning of the night	*9 10 11,12 100*
ICER	Transcriptionally regulated inhibitor	CRE	Inhibition of *Aa-nat, β_1-Adr, ICER, Per1* expression at the end of the night	*108 113 13 106*
DREAM	Posttranscriptional modifications Ca^{2+}-dependent protein–protein interaction with CREB	DRE CRE	Repression of *Aa-nat, β_1-Adr, Fra-2* gene expression during the day; competition with pCREB for CRE	*135* *134*
IEGs	Modification of action by homo/ heterodimer formation	AP-1	*Aa-nat* expression at the end of the night	
FRA-2			No effect on *Aa-nat* expression	*124*
Jun-B	Nocturnal increase in parallel to *Aa-nat*		Effects on *Aa-nat* not known	*126*
c-Fos				
Clock proteins		E-Box	Effects on *Aa-nat* expression not known	*140*
BMAL/CLOCK	Activator			
PERs/CRYs	Inhibitor			
CRX	Homeobox gene	GATTA-box	Maintainance of *Aa-nat* expression	*163*
OTX-2	Homeobox gene		Regulation of developmental/ differentiation of pinealocytes	*162*

inhibit melatonin formation, but that this inhibitory effect is much slower or smaller than the activating input of the NE pathway.

Additionally, it has been shown that NO affects the intracellular pathway of melatonin synthesis downstream of the ADR/AC/cAMP signaling route *(83)*. To date the functional role of the clearly observed effects of NO are not understood, but it is likely that this extremely rapid signal mediator might be an integrating intercellular messenger for all pinealocytes and other cells in the pineal gland *(87)*.

Background Music

For an increasing number of neurotransmitters/neuromodulators miscellaneous effects on pineal metabolism have been shown (for review see ref. *88*). Of these, at least some should be mentioned here. Two important neuropeptides, vasopressin and oxytocin, which are synthesized in the paraventricular nucleus, reach the rodent pineal gland by a monosynaptic connection to the pineal gland *(89)*. Vasopresin has been shown to potentiate the NE-induced accumulation of cAMP *(90)*, followed by AA-NAT activation *(91)*. Somatostatin and substance P have also been found in the pineal gland; however, their action as modulators of melatonin synthesis is not fully determined yet (for review see ref. *88*).

Apart from neuropeptides, the biogenic amines serotonin and dopamine, as well as the amino acid GABA have been found to be involved in regulation of melatonin biosynthesis (for review see ref. *88*). Serotonin, which is considered as substrate in melatonin synthesis *(25)*, appears to be also a secretory product of the pineal gland *(92–94)*, as well as a factor that potentiates melatonin release *(94,95)*. Understanding the role of these at most modulatory neuroactive substances in the rodent pineal gland is difficult, they have to be seen as part of an orchestra, by itself not necessary for rhythmic melatonin synthesis, but possibly demanded upon specific physiological situations.

Transcriptional Regulation of Melatonin Synthesis

In rodents, the induction of the *Aa-nat* gene expression is a prerequisite of the nocturnal increase of melatonin levels in the pineal gland (Table 1). During the light phase, transcripts of the *Aa-nat* are not detectable *(11,96–98)*, whereas the nocturnally increased release of NE induces a more than 100-fold increase in *Aa-nat* mRNA levels. This increase is followed by a rise in the AA-NAT protein levels within 2–3 h and is accompanied by an elevated AA-NAT enzymatic activity *(11,96,98)*.

CREB, the Drum Beat for Transcriptional Activation

As an initial step upon β_1-ADR stimulation in rodent pinealocytes, elevated cAMP levels activate two subtypes of the protein kinase A (PKA), the PKA type I and type II *(12)*. However, it has been determined that only the PKA II is important for the subsequent phosphorylation of the activating TF CREB at Ser 133 *(99)*, as it is only this subtype that translocates into the nucleus *(11,12,100)*.

The functional role of PKA I in pineal gland is not clear; however, it might keep cytosolic target proteins, like AA-NAT, in a phosphorylated state *(101,102)* and mediate the cAMP-induced inhibition of proteasomal degradation of AA-NAT *(8)*.

The phosphorylation of CREB at the Ser 133 *(103)* leads to homodimerization of pCREB and its binding to a co-activator, the CREB binding protein (CBP) *(103)*. The transcriptional complex pCREB/CBP enhances by binding to the CRE in the *Aa-nat* gene promoter gene expression at night *(104)*. This complex is also involved in the control of the clock gene *Per1* (see below) *(105)*, the β_1-*Adr* (see below) *(106,107)*, and the transcription factor (TF) *Icer* (inducible cAMP early repressor) (see below) *(108)*. The transcription of all these cAMP-inducible genes shows a pattern that parallels that of the *Aa-nat* mRNA accumulation *(11,106,109)* and that supports the idea of a common regulatory mechanism via the cAMP signaling pathway.

In addition to Ser 133, CREB has a phosphorylation site on the Ser 142 targeted by the calcium/calmodulin-dependent kinase II α (CaMKII) *(110)*. The physiological importance of this second phosporylation site was demonstrated for synchronization mechanisms of the circadian clock in the SCN, where maximal phase shifts of *Period1* (*Per1*) clock gene after a light pulse were only achieved upon CREB double phosphorylation *(111)*. Whether CREB phosphorylation at Ser 142 also occurs in the pineal gland has not yet been investigated.

Several mechanisms are involved in the termination of cAMP-induced gene expression in the rodent pineal gland. They are complex and include transcriptional as well as posttranscriptional processes. Reduction of pCREB has been shown in vivo to precede the decline of *Aa-nat* mRNA levels at the end of the night *(11)*. In vitro observations support the close relationship between NE-stimulation and the levels of pCREB by the observation that the amount of pCREB decreases within 30 min after NE withdrawal *(112)*. These studies demonstrated that the serine/threonine protein phosphatase, type I (PP1), is involved in down-regulation of pCREB levels possibly via translocation of the catalytic subunits of PP1 into the nucleus of the pinealocytes. This translocation process coincides with the decline in pCREB levels, approx 8 h after the beginning of the adrenergic challenge *(112)*. This direct regulatory link is confirmed by the fact that inhibition of PP1 abolishes the effects observed after NE removal, i.e., CREB remains phosphorylated, *Aa-nat* mRNA levels stay elevated, and no reduction in the amount and activity of the AA-NAT and in melatonin concentration occurs *(112)*.

What Goes Up, Must Go Down— the Ritardando of Melatonin Synthesis

The effects of CREB dephosphorylation on the down-regulation of melatonin synthesis are in concert with the inhibitory influence of the TF ICER, a member of the CREB/CREM family. As the night progresses, ICER accumulates in rodent pinealocytes *(108)*. The nocturnal increase of the ICER protein levels depends on the NE-stimulated activation of the *Icer* gene, which encodes four different splice variants, all of which contain the DNA binding domain, but lack the amino terminal phosphorylation domain, characteristic for activating transcription factors of the CREB/CREM family *(108)*. Therefore, the ICER protein levels solely determine the activity of its transcriptional repressor functions *(108)*. *Icer* mRNA expression shows significant diurnal variations in vivo, with almost undetectable amounts during the day and a dramatic increase at the beginning of the night reaching maximal level 4–6 h before the next light period *(11,13,108)*. Unlike the large fluctations in mRNA transcripts, day/night differences in the level of the ICER protein are rather shallow (three to five times difference), and ICER is also present during the day time *(11)*. Studies using CREM-knockout animals *(113)* and in vitro experiments with ICER antisense oligonucleotides *(11,114)* have demonstrated a desinhibition of *Aa-nat* transcription upon silencing of this TF. Notably, the phosphorylation of CREB seems to be unchanged in pinealocytes transfected with an *Icer*-antisense construct. In subsequent studies, it was demonstrated that the ratio between pCREB and ICER controls not only levels of *Aa-nat* gene expression, but also of the β_1-*ADR (106),* the clock gene *Per1*, and even of *Icer* itself *(11,106,108)*. The importance of the pCREB/ICER ratio for a temporally regulated gene expression is supported by the observation that the ontogenetic appearance of a rhythm in *Icer* abundance *(115)* is temporally linked to the onset in regulation and rhythmicity of *Aa-nat* and β_1-*ADR* mRNA levels *(106,116)*. Therefore, a generalized mechanism can be suggested for the circadian regulation of cAMP-sensitive genes in the rodent pineal gland, which all depend on a shift in the level of CREB phosphorylation and the amount of ICER protein and their competition for the binding to CREs of cAMP-inducible genes *(11,13,51,108,113)*.

On top of this robust transcriptional regulation of the *Aa-nat* gene, supplementary mechanisms shape the proper dynamics in melatonin synthesis. Among these are the PKA-dependent phosphorylation processes that stabilize nocturnal levels of AA-NAT activity. The PKA-dependent phosphorylation of the AA-NAT allows for the reversible binding of the chaperon protein 14-3-3, thus preventing ubiquitination and degradation of AA-NAT by proteasomal proteolysis *(117)*. This process *(8)* is the main mechanism controlling melatonin synthesis in ungulates, where the *Aa-nat* gene is constitutively expressed and continuously degraded *(14,101,102,118)*. In ungulates, it is only upon NE stimulation at night time that the proteasomal degradation becomes inhibited. In rodents, proteasomal proteolysis of AA-NAT is responsible for the rapid inhibition of melatonin synthesis observed when an animal sees the light during the dark

period. The importance of this mechanism for the termination of rodent melatonin synthesis under natural photoperiod condition is not yet fully clarified.

An additional mechanism for the timed regulation of melatonin synthesis in the rodent pineal gland is the transcriptional and post-translational regulation of the initiating receptor itself, the β_1-ADR: Levels of the *β_1-Adr* mRNA in the rat pineal gland show a circadian rhythm in parallel with *Aa-nat* mRNA, with regulation directly linked to the release of its own ligand, NE *(106)*. However, the transcription of the *β_1-Adr* gene is not followed directly by an incorporation of the receptor protein into the membrane, because the number of NE binding sites becomes elevated at the earliest 7 h after the peak of mRNA and is higher during the day than at night *(106)*. This phenomenon can be explained by a temporal sequestration of the receptor protein. It is only upon the lack of ligand during daytime that the β_1-ADR is incorporated into the membrane. A similar regulatory mechanism seems to hold true in the rat pineal gland for the α_1-ADR *(119)*. ADRs become desensitized under prolonged NE-stimulation and, as in the other tissues *(120, 121)*, this mechanism may contribute to the inhibition of cAMP/PKA/pCREB pathway by the end of the night.

Immediate Need for a Change in Tempus?

An important common mechanism for stimulus-induced gene expression in neurons is the activation of so-called immediately early genes (IEGs) *(122)*. They form the activator protein-1 (AP-1), a transcriptional regulatory complex, which interacts with the palindromic consensus sequence of the serum response element (SRE) in the gene promoter region. AP-1 is composed of homo- and heterodimers of members of the FOS and JUN family, including FRA-2 (Fos-related antigen 2) *(123)*. Their expression in the rat pineal gland is regulated by adrenergic stimulation *(124, 125)*, with *c-fos* and *junB* mRNAs rapidly and transiently increasing at the beginning of the night and decreasing later the night *(126,127)*. In the rat pineal gland, AP-1 activity and binding exhibit a circadian rhythm that occurs in parallel with the increase in *Aa-nat* transcription *(124,128)*. Notably, the translational product FOS appears in rat pineal gland much later, i.e., only 5 h after the peak in mRNA *(129)*, which is in contrast to the rapid sequence of these events observed in other systems.

A particularly well-investigated IEG is FRA-2, a major component of the AP-1 complex *(123,124)*. *Fra-2* expression follows a pattern similar to *c-fos* expression, with a fast increase of mRNA levels and delayed peak protein levels. FRA-2 was initially suggested to take part in inhibition of *Aa-nat* gene expression *(102)*. This concept was ruled out by experiments with transgenic rats expressing a dominant negative form of the *Fra-2* gene *(130)*, which did not alter dynamics of *Aa-nat* or *Icer* mRNA.

It has to be pointed out that there still exists a major gap in information about the functional role of IEGs in the rodent pineal gland, particular as the mode of action and the targets remain enigmatic. It may be speculated that AP-1 participates in other transcriptional events such as diurnal regulation or modulation of the other rhythmically expressed genes that have been found in the pineal gland *(131)* and, in this way, modulate rhythmic melatonin biosynthesis *(115)*.

DREAM-ing into the General Tune

Changes in Ca^{2+} levels are involved in multiple signaling events in rodent pinealocytes *(1,132)*. DREAM (downstream regulatory element [DRE] antagonist modulator) and its closely related potassium channel interacting proteins (KChIP) belong to a group of neuronal calcium sensors, which repress transcription in a Ca^{2+}- and cAMP-dependent manner *(133,134)*. Analyzing the promoter region of various pineal genes inducible by calcium stimulation revealed the presence of a DRE in the *Aa-nat*, *Icer*, and *Fra-2* gene. In follow-up studies it was shown that the DREAM/KchIP complex is indeed able to repress basal and induced transcription of *Aa-nat*, *Icer*, and the *Fra-2* genes *(135)*. Interestingly, in the rat pineal gland and retina, the endogenous binding activity of DREAM to the DREs shows day–night oscillations that are preserved under constant darkness *(135)*. The highest binding affinity to the DRE occurs during the day, coinciding with the lowest expression levels of *Aa-nat*, *Icer*, and *Fra-2*. Consequently, the decrease of binding to the DRE occurred with the onset of the dark period and resulted in the de-repression of gene transcription. As DREAM protein levels are unaltered over the day/night cycle, these obvious changes in binding seem to be related to a posttranscriptional modulation of DREAM/KChIP, caused by variation in the intracellular Ca^{2+} levels *(135)*. Additionally, the Ca^{2+}-dependent protein–protein interaction of DREAM–CREB is generally able to displace pCREB from a CRE *(166)* and to prevent CBP recruitment by pCREB. These results led to the conclusion that DREAM interacts with rhythmic transcription in the rodent pineal gland by two mechanisms, e.g., directly, by repression of DRE-containing genes, and indirectly, by scavenging away the activator pCREB *(135)*.

Clock Genes in the Pineal Gland, or Is a Metronome Needed?

In the SCN, clock genes and their protein products form a self-sustained transcription/translation loop by positive (*Bmal, Clock*) and negative (*Per1, 2,* and *3, Cry1* and *2*) TFs that regulate the endogenous rhythm and clockwork-dependent output signals *(136)*. In addition to the central clock in the SCN, "peripheral clocks" were found in all tissues investigated thus far *(137,138)*. All clock genes have also been found in the pineal gland of mouse (Fig. 2) *(139,140)* and

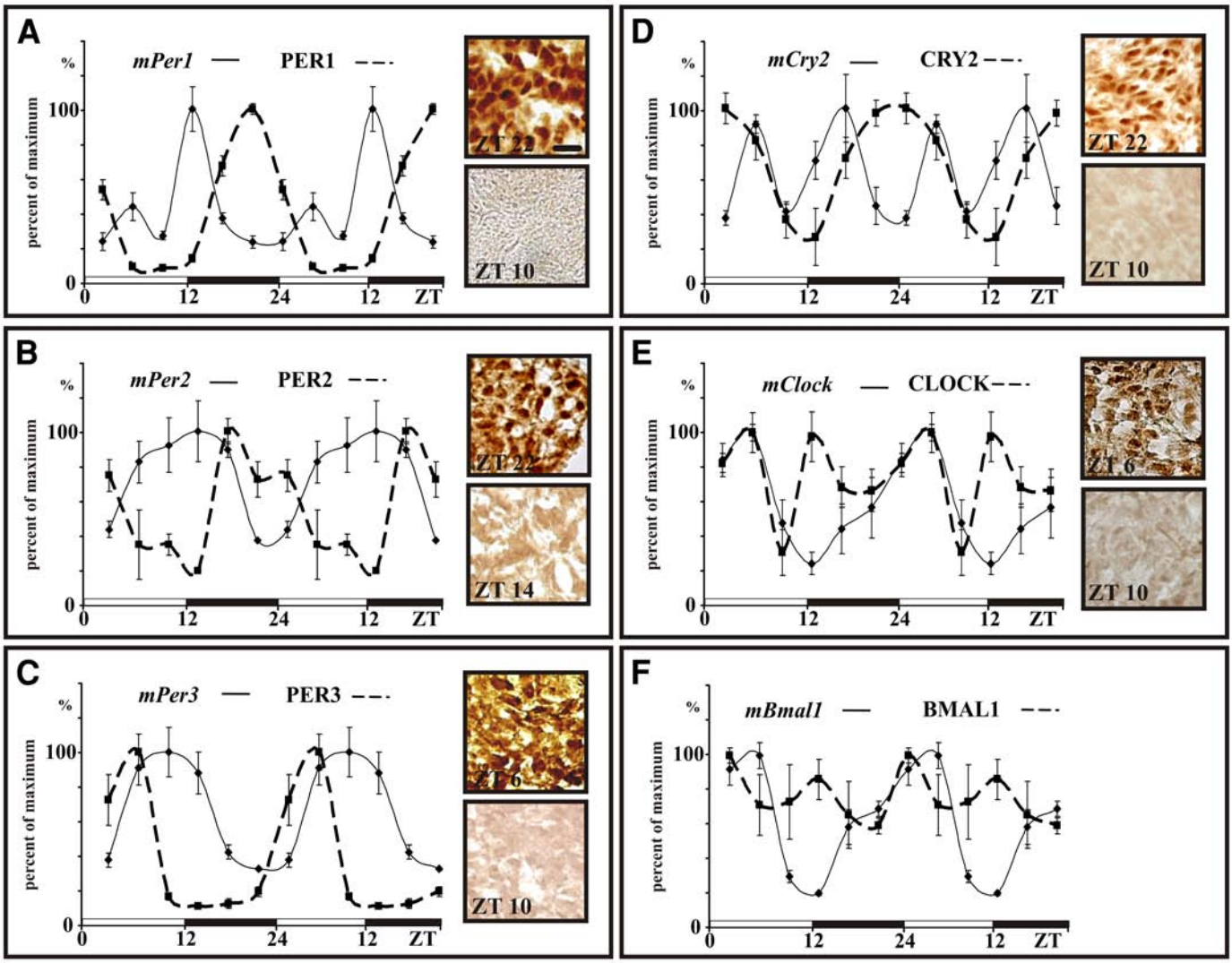

Fig. 2. Clock gene mRNAs and protein levels in the mouse pineal gland. Each graph represents a double plotted diurnal pattern of clock gene mRNA expression (solid lines) and of the corresponding protein products (dashed lines); (**A**) *mPer1*/PER1; (**B**) *mPer2*/PER2; (**C**) *mPer3*/PER3; (**D**) *mCry2*/CRY2; (**E**) *mClock*/CLOCK; (**F**) *mBmal1*/BMAL1. Representative immunohistochemical images of peak and trough values for clock gene product levels are given on the right hand side of each graph. Scale bar in (**A**) 10 μm (for all microphotographs). Adapted from ref. *140*.

rat *(109,141,142)*. In the mouse pineal gland, the time difference between the peaks in clock gene mRNA and protein is similar to that in the SCN *(2,136,140)*. However, the respective peak and though values in the pineal are 6–8 h phase delayed, as compared to the SCN *(140)*. In vitro studies using *Per1*-promoter::luciferase transgenic mice demonstrated that the pineal gland is not able to maintain a self-sustained rhythm, and in vitro rhythmic expression dampens out without external input *(137)*. A recent approach used *Per2*::luciferase transgenic mice, in which the posttranscriptional processes, necessary for proper functioning of clock proteins, are preserved. In different tissues of these mice a rhythmic expression of the *Per2* gene persisted for at least 20 d in the culture *(138)*, implying that the same holds true for pinealocytes. Closer examination of this system and studies with fibroblasts transfected with the *Per2*::luciferase construct indicate that the observed in vitro cessation of the rhythmic clock genes expression, reflects a gradual desynchronization of individual oscillator cells *(138,143)*. Resynchronization of these individual oscillators can be obtained by medium change or by chemical stimulation *(138,143)*. This might reflect processes that also occur in vivo, i.e., neuronal or/and humoral signals from the SCN to the pineal gland might coordinate rhythmic activity of pinealocytes. Whether the observed dampening of *Per1*::luciferase signals in vitro is due to desynchronization of cells lacking an input from the SCN is still to be investigated *(138)*. It can be envisaged that it is the nocturnal NE increase that synchronizes the expression of the *Per1* mRNA in individual pinealocytes *(139)*. Confirmatory, *Per1* mRNA expression was shown to rise at the onset of night in parallel to the accumulation of *Aa-nat* mRNA

transcripts *(109,139,144)*, both of which depend on the adrenergic signaling. In addition, a rhythmic *Per1* expression in the pineal gland of ganglionectomized rats *(144)* can be restored by treatment with the β-adrenergic agonist isoproterenol *(144)*, and in vitro inhibition of PKA activity or CREB phosphorylation abolishes induction of *Per1*/PER1 *(139)*.

The sensitivity of other clock genes to the NE stimulation was also investigated in the pineal of rat *(142,144)* and mouse (Karolczak, personal observations). Although expression of *Per2* expression is diminished in ganglionectomized rats, it cannot be restored by stimulation with β-adrenergic agonists, suggesting a different control mechanism, as compared to *Per1 (144)*. This suggestion is supported by observations that the *Per2* transcription starts to increase before the light/dark transition.

In the rat pineal gland the clock gene *Cry2* has also been shown to be regulated by NE *(142)*; however, this observation could not be confirmed in mice (unpublished results).

Taken together it seems that the archaic function of clock genes as TFs is put in the SCN in the special context of rhythm generation. In "peripheral" or more precisely SCN-distal tissues, the clockwork requires an input signal to oscillate. Apparently, the *Per1* gene is the pivotal trigger to start this oscillation, as exemplified at least in the pineal gland and in the pars tuberalis *(22,139)*. Interestingly, *Per1* appears to trigger the light-induced phase resetting of the central clock, which is also mediated via a pCREB-induced *Per1* transcription *(2)*.

Rhythmic Genes, Genes in Rhythm

For an increasing number of genes, rhythmic expression is demonstrated in the rodent pineal organ. Using cDNA array analysis 38 genes were identified, whose expression exhibit a day/night differences in the rat pineal *(131)*. One of these, Id-1, is a helix-loop-helix (HLH) *(145)* protein that is known to interact with basic (b) HLH proteins *(146)*, such as *Per1* and *2*. This interaction prevents binding to the E-box and in consequence decreases transcription of E-box–containing genes *(147)*. The nocturnal induction of Id-1 in the pineal is associated with an increase of its heterodimeric nuclear complexes that might be associated with changes in DNA binding activity *(131)*.

The mRNA level of another TF, the orphan nuclear receptor NGFI-B, is also at an elevated level during the night and this rise is accompanied by an increased protein level in the nucleus of pinealocytes at night *(148)*. In the pineal gland, NGFI-B is under the control of an NE/cAMP mechanism that probably involves action of the FRA-2–enriched AP-1 complex (see above), which binds to the promoter region of the *Ngfi-B* gene *(148)*. Potential targets of NGFI-B in the pineal gland are currently undefined, but would not include the *Aa-nat*, as it lacks a NGFI-B response element *(104)*.

Microchip analysis revealed rhythmic expression of the mitogen-activated protein kinase phosphatase-1 (MKP-1) *(149)*, which is an important regulator of MAPK (mitogen-activated protein kinase) activity in multiple physiological processes *(165)*. MKP-1 expression has been shown to be regulated by both α_1- and β_1-adrenergic stimulation *(149)*. Substrates of MKP-1, such as MAPK, can influence AA-NAT activity *(149,150)*.

The microarray analysis also revealed a 60-fold night/day difference in the abundance of PEPT1 *(151)*, which is a member of the 12-transmembrane spanning domain transporters. Its expression is regulated by the NE/cAMP-dependent pathway. Although the role of PEPT1 in the pineal gland is not clear, its high expression during the night suggests that it may contribute to the regulation of circadian pineal physiology by clearance of active or degraded neuropeptides *(151)*.

The methionine adenosyltransferase (MAT) synthesizes (*S*)-adenosylmethionine, which is the methyl donor for the HIOMT, the enzyme of the last step in melatonin synthesis. MAT is specifically expressed in the rat pineal gland during night and transcriptionally regulated via the NE/cAMP pathway *(152)*. This is of particular interest, as melatonin production might be altered through changes in MAT activity, without accompanying changes in AA-NAT activity. MAT may be added to the genes regulated via the transcription factors CREB and ICER.

In a differential display screen, a circadian expression of the nocturnin gene was discovered in the *Xenopus leavis* retina and was suggested to be involved in melatonin synthesis *(153,154)*. In the mouse, nocturnin expression has been found in multiple tissues, including the retina, spleen, liver, and pineal gland *(155)*. In all tissues, apart from the pineal gland, which has not been examined, nocturnin follows a rhythmic pattern of expression, with a high level during the dark period *(155)*. At present, it is not known whether nocturnin is coupled to physiological rhythms in the rodent pineal gland; however, its wide expression pattern suggests that its function is not restricted to the melatonin synthesis *(155)*.

Development Dynamics, Are Sounds of the Overture Preserved?

The nocturnal production of melatonin by the pineal gland is a conserved process present in all vertebrates, although mechanisms controlling this process developed differently during ontogeny. Despite the fact that in the course of evolution the mammalian pineal gland has lost its direct photosensitivity *(1,156)*, some components of the phototransduction cascade are well preserved in mammalian pinealocytes *(157,158)*.

These molecular similarities between the two diencephalic derivatives—pineal gland and retina—were a basis for a quest of common genes that could be involved in the development of both. The ciliary neurotropic factor (CNTF), which

was previously reported to inhibit the development of the rhodopsin-positive photoreceptors in rat retinal cultures *(159)*, was shown to be expressed in the pineal gland of newborn mice and to reduce in a dose-dependent manner the number of rhodopsin-immunoreactive cells in the pineal gland *(160,161)*, probably by suppressing the proliferation of a particular type of pineal progenitor cells or the expression of photoreceptors. This effect seems to be specific for CNTF, because other neurotrophins such as TGF-α exerted an inhibitory effect on rod development only in retinal cultures *(161)*.

A key molecule involved in the cell fate and the development of the pineal gland is the homeobox gene *Otx2*, as in a conditional knock-down mouse a complete loss of pinealocytes and a conversion of retinal photoreceptors into amacrine neurons has been observed *(162)*. Otx2 is an upstream regulator of another member of the homeodomain genes, the *Crx*. Although $Crx^{-/-}$ animals do not show gross abnormalities of pineal gland structure, the expression of the *Aa-nat* is abolished *(163)*. Interestingly, in addition to its expression during the development, CRX shows a rhythmic, NE-dependent expression in the adult pineal organ *(164)*, with a peak prior to the elevated *Aa-nat* expression *(164)*.

Another member of the *Otx* homeobox genes family, *Otx5*, has been found to be crucial for the rhythmic expression of *Aa-nat* in the zebrafish pineal complex *(165)*; however, the extent of the involvement of this gene in the regulation of MEL synthesis in rodents has not yet been investigated.

Echos and Inspiration

Melatonin represents a precisely timed hormonal message of darkness and is an important output signal of the circadian system whose functional importance is particularly evident in photoperiodic animals: they have to read the melatonin signal to time the appropriate period for reproduction in accordance with environmental conditions. Deciphering the regulatory molecular mechanisms behind melatonin production is therefore required to understand nature's strategy for disseminating zeitgeber cues. Different ways of generating the melatonin message of darkness have evolved across species *(14)*. In rodents, a precisely tuned concert of input signals leads to a transcriptionally conducted music that shows a clear-cut crescendo of gene expression at the beginning of the night, and a decrescendo that starts, notably, before the end of the night. The actual informational content of this simple hormonal message depends on the phase of the rhythms of day and night, season and life. Present ever since the dawn of life, the solar cycle with darkness as part of it is the most powerful external cue and selecting force for photosensitive organisms. Our genes have experienced more about it than we currently understand, but surely, in the days of the genomics, the dark truth will be enlightened.

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